

Water quality and antifungal resistance of yeast species from selected rivers in the North West Province

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Philippians 1:6

Being confident of this, that he who began a good work in you will carry it on to completion until the day of Christ Jesus.

Proverbs 3:5-6

Trust in the Lord with all your heart and lean not on your own understanding; In all your ways submit to him, and he will make your paths straight.

Proverbs 16:3

Commit to the Lord whatever you do, and he will establish your plans.

Psalms 23

You anoint my head with oil; My cup runs over.

I dedicate this thesis to my wife, Nomathemba, my parents, T'seliso and Maradebe Monapathi, my brother and sister, Radebe and Busisiwe and my children, Lebohang, Miyakazi and Mashiya. I wish to pass my gratitude and appreciation for the love and support you gave to me through this hard challenging journey. Without you, this work would not exist. I really am blessed to have such encouragement and inspiration by my side.

Kea leboha matebele

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ABSTRACT

The present study focused on the diversity and characteristics of yeasts present in North West Province (NWP) surface water as well as antifungal agents and associated resistance. The first part of this study is a review that addressed the diversity, significance and health implication of aquatic yeasts. The review highlighted the presence of diverse yeast species in aquatic environments. It detailed characteristics of yeasts which could be beneficial or detrimental in human, plants and animals. A gap in research between clinical isolates, known pathogens, and environmental isolates was emphasized. This is vital as some studies have shown similar phylogenetic relationships between clinical and environmental isolates. In the second part of the study, the application of yeasts as water quality indicators was discussed. From four selected rivers in the NWP, significant differences in physico-chemical and microbiological parameters were observed seasonally as well as between the rivers systems. Furthermore, an association was observed between some physico-chemical parameters and yeast levels. High nutrient load, chemical oxygen demand and dissolved oxygen indicated eutrophication conditions in these river systems. Some studies have also associated high levels of yeasts and certain yeasts species with faecal contaminated water. Pathogenic yeasts were resistant to various antifungal agents. In the third part of the study, efflux pump genes (*CDR1*, *CDR2*, *FLU1* and *MDR1*) coding for resistance to fluconazole were detected in environmental *Candida albicans* isolated from the water resources. The sequences of these genes were phylogenetically similar to those from clinical origin. These findings were worrisome since *C. albicans* is an opportunistic pathogen that causes most infections in human immunodeficiency virus (HIV) patients and fluconazole is the most used antifungal agents in HIV treatment. The fourth part of the study addressed pollution from pharmaceutical products and yeasts in water. Yeast levels were determined from copy numbers of 26S

rRNA genes in environmental DNA and were quantified by qPCR. Commonly used antifungal agents were also quantified and screened for. The study provided an insight into yeast levels determined by rapid DNA extraction and a culture independent approach. Furthermore, antifungal agents were detected and fluconazole levels quantified. The information generated in this study demonstrated association of yeast levels to polluted water as indicated by physico-chemical parameters. Antifungal resistance among pathogenic yeasts as well as mechanisms of resistance was demonstrated. Additionally, the presence and levels of antifungal agents suggested that selection and maintenance of antifungal resistant yeasts occur in aquatic ecosystems. In general, the results from the present study will be valuable in understanding the impact of pathogenic yeasts in aquatic systems. It will be beneficial in making policies for ensuring that mitigation strategies are put in place to prevent spread of antifungal resistance from clinical to aquatic environments. The outcome from the results will contribute towards improved antifungal therapy and development of new strategies against antifungal resistance.

Keywords: Environmental yeasts, *Candida albicans*, antifungal resistance, antifungal agents, fluconazole resistance, surface water quality.

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CHAPTER 1

Introduction and Problem Statement

1.1 Water situation in South Africa (SA)

South Africa is faced with both quantitative and qualitative water challenges (DWS, 2015). The country is ranked as the 30th driest country in the world. Its location in a semi-arid part of the world makes it prone to episodic and sometimes enduring droughts (Kohler, 2016). Water availability across the country is also highly uneven because of poor spatial and temporal distribution of rainfall (DWS, 2015). The average rainfall for the country is about 450 mm per year (mm/a), well below the world average of about 860 mm/a (Kohler, 2016). High evaporation also reduces the availability of surface water (DWS, 2015).

Water resources in South Africa are dominated by surface water and the water is used for agriculture, mining, urban and industrial requirements (Roux et al., 2014, DWS, 2015). Agriculture, particularly irrigation is the country's largest water user sector. However, large quantities of water are needed for all water requirements; more than what is available in the country (DWS, 2015). Due to the water shortage, South Africa imports water from Lesotho. Lesotho Highlands Water Project through its dams (Katse and Mohale) and artificial lakes carries water into South African rivers. The mountain kingdom is the supplier of water to the Gauteng metropolitan area in South Africa (Rousselot, 2015).

The scarcity of water in the country is worsened by the deterioration in water quality that is due to pollution. The deterioration in water quality is mostly caused by industrial and mining effluents, runoff from agricultural activities and urbanisation (Oberholster and

Ashton, 2008). This is worsened by out-dated and insufficient water and sewage treatment plant infrastructure and unskilled operators at water treatment plants (CSIR, 2010).

1.2 Water challenges in the North West Province (NWP)

Water problems that face SA are also a reflection of water challenges in the NWP. Groundwater and surface waters are the main sources of water. World-wide, groundwater plays a pivotal role as a source to freshwater (Knüppe et al., 2011). It is used in agriculture, sanitation and for drinking purposes. However, groundwater quality is affected by mining and industrial effluents, waste disposal and agricultural runoff (Khatri and Tyagi, 2015). Surface water in the province is scarce because of non-perennial surface waters (NWP-SoER, 2014). Climate in the province varies from west to east. The mountainous eastern part is wetter from the rainfall and the western part is drier from the semi-desert plains (READ, 2015). A large proportion of the province is considered to be an arid region particularly in the west (NWP-SoER, 2014).

South Africa's water resources have been decentralized into 9 water management areas (WMAs) for water quality management reasons (DWS, 2016). These WMAs were established as a component of National Water Resource Strategy (DWAF, 2013c) by the National Water Act, 1998 (Act No. 36 of 1998) to protect, use, develop, conserve, manage and control water resources (Perret, 2002). Selected surface water resources in the present study are part of the Vaal Major system and some fall within the Limpopo WMA. The Mooi River, Harts River and Schoonspruit River form part of the Vaal Major WMA. Crocodile River and Marico River are located in the Limpopo WMA (DWS, 2016). These water resources are used for agriculture, mining, industrial, religious as well as recreational activities (NWP-SoER, 2014).

1.3 Microbial pollution

The presence of microorganisms in our water systems affects the water quality. These microorganisms include algae, bacteria, fungi, protozoa, viruses and bacteriophages (Azizullah *et al.*, 2011, Boyd, 2015) which are all part of the natural microbial community of the water. When water conditions change due to pollution by chemical substances and physical factors then the population dynamics of these microorganisms are affected (Fuhrman *et al.*, 2015).

Water resources can be contaminated with faecal originating matter that consists of hazardous chemicals and toxin producing as well as pathogenic microorganisms (Ponce-Terashima *et al.*, 2014). Bacterial species are mostly used as indicator organisms (Pereira, 2009, Adeleke and Bezuidenhout, 2011). However, studies by Hagler and Mendonca-Hagler (1981), Arvanitidou *et al.* (2002), Van Wyk *et al.* (2012), Monapathi *et al.* (2017) have presented evidence that linked yeasts as potential indicators of surface water quality.

The yeast density and diversity present in water could be used to determine the type and degree of water pollution. Clean or mildly polluted water have yeast counts ranging from few cells per litre to several hundreds. When the water is polluted or in the presence of algae, yeasts levels could reach a few thousand cells per litre (Hagler and Mendonca-Hagler, 1981). Non-fermentative yeast species are dominant in clean water while polluted water is mostly dominated by fermentative yeasts (Hagler and Ahearn, 1987). *Saccharomyces cerevisiae* is generally uncommon in clean habitats. Its presence in a water system is an indication of pollution (Hagler, 2006, Brandão *et al.*, 2010).

C. albicans naturally occurs as a commensal in mucosal oral cavity, gastrointestinal tract and genitourinary tract (Barnett, 2008). Its presence in faeces makes it a choice to complement bacteria when monitoring human faecal pollution of environmental source (Hagler, 2006). A significant correlation between yeast levels and faecal indicator bacteria was also observed in a study conducted by Arvanitidou et al. (2002). Dynowska. (1997) also conducted studies on yeasts in association with polluted water. The study concluded *Cryptococcus*, *Pichia* and *Rhodotorula* could be used as bio-indicators of pollution. Significant correlation between yeasts and faecal coliforms was observed in a study by Brandão et al. (2010). The dominant yeast species isolated were: *Candida krusei*, *C. guilliermondii* and *C. tropicalis*. These yeast species were associated with faecal water pollution by warm-blooded animals.

1.4 Pathogenic yeasts

Recent studies have demonstrated that several yeast species, some opportunistic pathogens, were frequently isolated from the surface water in the NWP (Van Wyk et al., 2012, Monapathi et al., 2017). This is a health concern especially to immunocompromised people who use the water for different purposes including contact or consumption. Pathogenic yeasts may cause mild to mortal infections. The most common yeast infection, candidiasis, is caused by *Candida* species especially *C. albicans*. It accounts for high mortality rates (60%) amongst HIV patients (Mayer et al., 2013, Bassetti et al., 2018).

Cryptococcus neoformans causes Central Nervous System (CNS) infections in patients with HIV/AIDS globally (Limper et al., 2017). HIV-associated cryptococcal meningitis death rates are estimated at 150000–200 000 deaths per year and these are mostly in sub-Saharan Africa (Jarvis et al., 2014).

1.5 Antimicrobial agents: environmental pollutants

Antifungal drugs are used in human and veterinary medicine and agriculture to treat and prevent fungal infections (Jampilek, 2016, Dalhoff, 2017). According to their mechanisms of action, antifungal drugs are classified into 4 different classes; azoles, polyenes, echinocandins and nucleoside analogues (Vandeputte et al., 2012). Antifungal agents are also provided as part of the prophylactic treatment of HIV patients and other yeast infections (Abrantes et al., 2014, Nett and Andes, 2015).

Antifungal agents, either as active compounds or derivatives are flushed into wastewater treatment plants (WWTPs). A portion is removed in these plants but a fraction subsequently land into river systems, normally at sub-therapeutic levels (Chen and Ying, 2015). Runoff from both animal and agricultural use also lands into environmental water. Antifungal agents have thus emerged as a new class of environmental pollutants (Singer at al., 2016). Relevant pathways for antifungal agents into the environment thus include municipal and industrial wastewater, veterinary and livestock as well as land application of manure and sludge (Singer at al., 2016).

1.6 Antifungal resistance

Antifungal resistance renders treatment difficult and contributes to the increased mortality rate. The situation is worsened in the immunocompromised members of the population (Srinivasan et al., 2014). Antimicrobial resistance is caused by continuous exposure, excessive and inappropriate use of antifungal agents (Carvalho and Santos, 2016). Antifungal resistance of yeasts from aquatic environments have been reported in South Africa and elsewhere (Medeiros et al., 2008, Brandão et al., 2010, Monapathi et al., 2017). Largely, increased yeast resistance to commonly used antifungals has been observed in

clinical isolates (Sanglard, 2016, Hrabovský et al, 2017, Canela et al., 2018). The mode of action and mechanism of antifungal resistance is similar in human, veterinary medicine and plant protection (Ribas et al., 2016). These resistance mechanisms may thus also be similar for yeasts from the environment.

1.7 Antifungal resistance mechanisms

The prolonged release of antibiotics into WWTPs is associated with the release of antibiotic resistance genes (Newton et al., 2015). Similar data are not available for antifungal resistance scenarios. Clinical studies at molecular level have been conducted in *C. albicans* to explain fluconazole resistance. These include overexpression of efflux pump genes such as multiple drug resistant (*FLU1* and *MDR1*) and *Candida* drug resistant (*CDR1* and *CDR2*), alteration of the drug target gene, *ERG11* and inactivation of the sterol C5.6-desaturase encoded for by *ERG3* gene (Cowen et al., 2015, Khosravi Rad et al., 2016, Salari et al., 2016, Sanglard et al., 2016). The aforementioned mechanisms have also been studied in non-*albicans Candida* species such as *Candida dubliniensis*, *C. glabrata*, *C.krusei*, *C. parapsilosis* and *Cryptococcus neoformans* (Lamb et al., 1995, Guinea et al 2006, Coleman et al., 2010, Souza et al., 2015, Bhattacharya and Fries, 2018). There are limited studies on environmental isolates and the present study focuses on efflux pumps in environmental *C. albicans* species.

1.8 Problem statement

Surface water in the NWP is used for mining, agricultural, industrial rural and urban sectors, full contact water sports, recreational activities as well as religious ceremonies (NWP-SoER, 2014). However, these anthropogenic activities negatively affect the water quality in the province. Chemical substances from mining and agriculture, faecal matter

from informal urban and rural areas, agricultural runoff and poorly or non-treated sewage from urban WWTPs pollute surface water in the province (Van Der Walt, 2002, NWDACERD, 2010, NWP-SoER, 2014). The majority of NWP WWTPs are either (i) not working optimally, (ii) not properly managed, (iii) working beyond the systems' design parameters or (iv) a combination of these (DWAF, 2013a). It is also known that even efficient and effective WWTPs only partially remove pharmaceutical products (Kümmerer, 2009). Nutrient loading from sources, including WWTP effluents threaten the water quality of the NWP and lead to eutrophication (Griffin, 2017). Bacteria and algal levels have been used to describe water quality in such polluted waters. From previous studies it was demonstrated that yeasts are also indicators of pollution and there are several pathogenic species that could be used as indicators of human faecal pollution as well as risk factors. Furthermore, it is known that a large proportion of the NWP population is HIV positive and on antiretroviral treatment regimes, that include prophylactic use of fluconazole (Johnson et al., 2017, Statistics S.A , 2017) There are, however, limited studies on (i) yeast levels and diversity in surface waters in South Africa (ii) linking this to general water quality and pollution (iii) antifungal resistance patterns of yeasts from surface water (iv) resistance mechanisms of the yeasts (v) presence of antifungal agents in surface water.

The aim of this study was to thus determine if there is interplay between water quality and antifungal levels as well as resistance of diverse yeast species from selected rivers in the NWP surface water

Specific objectives were to:

- 1) Determine the physico-chemical parameters and yeast levels of the water samples from selected NWP River systems.
- 2) Relate yeast levels and species to water quality parameters, with relevance to organic and inorganic substances that could cause selection for antifungal phenotypes
- 3) Compare culture dependent yeast levels and diversity to culture independent levels and diversity.
- 4) Study the mechanisms of resistance to fluconazole and related antifungal resistance using molecular methods.

1.9 Outline of the thesis

This thesis comprise of 6 chapters. Chapter 1, already presented is an introduction with problem statement, aims and objectives. Chapter 2 is a literature review and is presented as a review paper. Chapter 3 to 5 are presented in research based structures for individual publications. Some overlaps of information was thus unavoidable.

CHAPTER 2

This chapter reports on freshwater pollution globally and in the North West Province, where the study was conducted. The presence of yeast in water, their importance and implications as pollution indicators and disease causal agents is also addressed. The paper also discussed use of antifungal agents, antifungal resistance and its cause and possible resistance mechanisms

Title: Aquatic yeasts: Diversity, Characteristics and Potential health Implications

Authors: Monapathi M.E., Rhode O.H.G. and Bezuidenhout C.C.

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Manuscript number: WR46856

CHAPTER 3

The chapter provides an overview on the current state of surface water systems in the North West Province. It further informs on the use of yeasts as water quality indicators. The paper reports on the presence of pathogenic yeasts as disease causal agents to people who use the water directly. Antifungal resistance shown by some of the isolated

pathogenic yeasts is a global concern and a public health threat and the paper clearly discussed the matter.

Title: Physico-chemical levels and culturable yeast diversity in surface water: A consequence of pollution

Authors: Monapathi M.E., Rhode O.H.G. and Bezuidenhout C.C

Target journal: Water Science and Technology

CHAPTER 4

The chapter describes the fluconazole resistance by the most important pathogenic yeast, *Candida albicans* determined by disk diffusion method. The presence of efflux pumps that are responsible for resistance are determined by end-point PCR in *C.albicans* isolated from NWP surface water. Additionally, phylogenetic analysis is conducted to compare sequence similarity between clinical and environmental *C.albicans* efflux pump genes. High sequence similarity was observed between clinical and environmental isolates.

Title: Efflux pumps genes of clinical origin are related to those from fluconazole resistant *Candida albicans* isolates from environmental water

Authors: Monapathi M.E., Rhode O.H.G. and Bezuidenhout C.C.

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CHAPTER 5

The chapter provides an insight into river water pollution from antifungal resistant pathogenic yeasts. The chapter employs culture independent methods in determining yeast levels in surface water. Quantitative PCR analysis was used to quantify yeasts from environmental DNA. The chapter also outlines how antifungal levels were analysed using solid phase extraction. The extracts were analysed with liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer. The chapter links exposure of yeasts to antifungal drugs in water to yeasts resistance. Some of the yeasts in water are pathogens and antifungal resistance is a public health concern.

Title: Yeast and antifungal drugs levels from polluted surface water: perspective on antifungal resistant yeasts

Authors: Monapathi M.E, Horn S., Vogt T., Gerber E., Pieters R., Bouwman H., Rhode O.H.G. and Bezuidenhout C.C

Target Journal: Journal of Water and Health

CHAPTER 6

It provides significant conclusions to the present study. Recommendations for future research are provided.

CHAPTER 2

Aquatic yeasts: Diversity, Characteristics and Potential health Implications

2.1. Introduction

Yeasts are eukaryotic microorganisms classified in the kingdom fungi and consist of two phylogenetic groups i.e. Ascomycetes and Basidiomycetes. Ascomycetous yeasts produce ascospores within a naked ascus, whilst Basidiomycetous yeasts form basidiospores outside the basidium (Kurtzman and Fell, 2011). Macroscopically, the yeasts can be divided into two groups based on their colony pigmentation using Diazonum Blue B (DBB) test on at least several solid growth media used (eg skim milk agar, urea agar and YNB agar) (Hagler and Ahearn 1981). Basidiomycetous yeasts comprise of species that produce pink, salmon or reddish colonies, with the exception of a few cases. Members of yeast species that form white or cream-coloured colonies are both classified into the Ascomycota and in the Basidiomycota (Gadanhó et al., 2003).

Yeast identification was initially based on morphological and physiological traits (Woollett and Hendrik 1970, Rosa et al., 1995, Sláviková and Vadkertiová 1997, Bogusławska-Was and Dabrowski, 2001). This identification is laborious, strenuous and in many cases inconclusive (Kurtzman and Robnett, 1998). Such an approach is not reliable on its own for identification (Rodrigues et al., 2018). On the other hand, some of the studies that identified yeasts have solely used molecular tests (Gadanhó and Sampaio 2004:2005, Pincus et al., 2007, Aguilar et al., 2016, Romão et al., 2017). However, combining both morphological and molecular analyses has increased the reliability of identification (Vaz et

al. 2011, Brandão et al., 2011:2017, Brilhante et al., 2016, Rodrigues et al., 2018, Novak Babič et al., 2016, Araújo et al., 2017, Monapathi et al., 2017).

Some recent studies have applied Next Generation Sequencing (NGS) methods to identify and genetically characterize yeasts (Wilkening et al., 2013, Aguilar et al., 2016, Okuno et al., 2016, Novak Babič et al., 2016, Romão et al., 2017). When using this technique, specific barcode sequences of yeast species in environmental DNA could be targeted. Venter and Bezuidenhout (2016) made arguments for the use of barcode DNA markers to study aquatic ecosystems. In this approach, direct isolation of DNA from water sources is conducted and using multiple primer sets researchers could get community diversity data of several taxa that are present or were recently present in the habitat (Venter and Bezuidenhout, 2016). Direct sequencing/NGS technologies have been used successfully in bacterial community structure and dynamics in fresh water environments (Zarraonaindia et al., 2013, Jordaan and Bezuidenhout, 2013:2016). For yeast studies, NGS technology has not been explored.

Yeasts are closely linked to daily activities such as culture, economy and nutrition (Barriga et al., 2011). In industries, yeasts are mostly used in fermentation processes. *S. cerevisiae* have been used for the production of fermented foods and beverages (Barriga et al., 2011) and production of electricity in microbial fuel cells (Schaetzle et al., 2008). Yeasts species such as *S. cerevisiae*, *Scheffersomyces stipitis*, *Schizosaccharomyces pombe* and *Pichia fermentans* are used in the production of ethanol for the biofuel industry (Azhar et al., 2017). Secondary metabolites such as enzymes, vitamins, capsular polysaccharides, carotenoïds, polyhydric alcohol, lipids, glycol lipids, citric acid, ethanol and carbon dioxide

are also produced by yeasts (Venkatesh et al., 2018). In agriculture, vegetable producers use yeasts as biological control agents to manage plant pathogenic fungi that attack vegetable crops (Punja and Utkhede, 2003). Yeasts can also be used as a bio-fertilizer. This application has received substantial attention because of yeasts bioactivity and safety for human and the environment (Agamy et al., 2013). These activities are associated with processes requiring large quantities of water and in-turn release comparable or even larger quantities into treatment and disposal aquatic systems.

Table 1 provides a summary of some yeast studies conducted globally. The following continents and their respective countries were in the study: Africa (Egypt, Ethiopia, Cameroon, Nigeria, South Africa and Zimbabwe), Asia (China, Japan, India, Iran, Pakistan, and Korea), Europe (Germany, Poland, Portugal, United Kingdom, Scotland, Slovakia, and Slovenia), North America (Brazil, Canada), South America (Colombia, Argentina) and Oceania (Australia). Both clinical and aquatic studies are addressed. The background to the study, stipulated in the table show that aquatic studies have mostly reported on the diversity and yeasts as organic pollution indicators. Studies conducted from clinical isolates have reported on yeasts diversity, antifungal resistance, gene expression and resistance mechanisms. Environmental studies have also reported on the occurrence and antifungal resistance. It is interesting to note that clinical and environmental isolates possess similar antifungal resistance and mechanisms. The presence of virulence factors from clinical and environmental isolates is also highlighted in the table. The methods of yeasts classification and identification from both resources are similar.

Table 1. Some of the aquatic environment and clinical studies conducted on yeasts

Authors	Background to study	Resource type	Country	Mode of identification	Asco/Basidiomycota	Antimicrobial activity	Resistance mechanisms	Virulence
Environmental studies								
Woollett and Hendrik, 1970	Pollution	Freshwater	America	Morphology, physiological tests	Both	-	-	-
Hagler and Medonca-Hagler, 1981	Pollution	Marine , estuarine waters	Brazil	Morphology, physiological tests	Both	-	-	-
Rosa et al., 1995	Pollution	Lake	Brazil	Morphology, physiological tests	Both	-	-	-
Dynowyska, 1997	Pollution	River	Poland	Morphology, physiological tests	Both	-	-	-
Sláviková and Vadkertiová 1997	Pollution	River	Slovakia	Morphology, physiological tests	Both	-	-	-
Bogusławska-Was and Dabrowski, 2001	Pollution	Lagoon	Poland	Morphology, physiological tests	Ascomycota	-	-	-
Nagahama et al., 2001	Diversity	Marine	Japan	Morphological, physiological and molecular methods	Basidiomycota	-	-	-
Gadanho et al., 2003	Diversity	Marine	Portugal	DNA fingerprinting	Both	-	-	-
Libkind et al., 2003	Diversity	Lakes, lagoons and rivers	Argentina	Physiological tests, DNA fingerprinting	Both	-	-	-
Gadanho and Sampaio	Diversity	River	Portugal	PCR, Sanger sequencing	Both	-	-	-

2004										
Gadanhó and Sampiao,	Diversity	Marine	Portugal	DNA fingerprinting, Sanger sequencing	Both	-	-	-		
2005										
Yamaguchi et al., 2007	Diversity	Bottled and tap water	Brazil	Physiological tests, molecular methods	Ascomycota	-	-	-		
Medeiros et al., 2008	Diversity	Natural lakes , rivers	Brazil	Physiological tests, PCR, Sanger sequencing	Both	Done	-	-		
Pereira et al., 2009	Diversity	Drinking water, surface water, spring water, and groundwater	Portugal	Morphological, physiological tests	Both	-	-	-		
Biedunkiewicz and Baranowska, 2011	Diversity	Surface water	Poland	Morphology, physiological tests						
Brandão et al., 2010	Diversity	Lakes	Brazil	Physiological, Sanger sequencing	Ascomycota	Done	-	-		
Vaz et al., 2011	Diversity	Marine and lake sediments, marine water and freshwater from lakes	Antarctica	Morphological, physiological tests, PCR fingerprinting , Sanger sequencing	Both					Extracellular enzymatic activity
Brandão et al., 2011	Diversity	Lakes	Brazil	Morphology, physiological tests, DNA fingerprinting, Sanger sequencing	Both	-	-	-		

Ayanbimpe et al., 2012	Diversity	Wells, streams, taps, boreholes	Nigeria	Morphology, physiological tests	Both	-	-	-
Van Wyk et al., 2012	Diversity	Rivers	South Africa	Morphology, physiological tests	Both	-	-	-
Krause et al., 2013	Diversity	Marine	Germany	Physiological tests, molecular methods:	Both	-	-	-
Samah et al., 2014	Diversity	Groundwater	Egypt	Morphology , physiological tests	Ascomycota	-	-	-
Silva-Bedoya et al., 2014	Diversity	Lakes	Colombia	Morphology, DNA fingerprinting, Sanger sequencing	Both			
Novak Babič et al., 2016	Diversity	Tap and groundwater	Slovenia	Physiological tests, molecular methods: NGS	Both	-	-	-
Aguilar et al., 2016	Diversity	Oil sands, tailings, ponds, sediments and surface water	Canada	NGS	Both	-	-	-
Brilhante et al., 2016	Resistant mechanisms	Lake	Brazil	Morphology , physiological tests	Both \	Done	Done	-
Romão et al., 2017	Diversity	Beach sands	Portugal	Sanger sequencing, NGS	Both	-	-	-
Brandão et al., 2017	Diversity	lakes	Brazil	Morphology, physiological tests, Sanger sequencing	Both	-	-	-
Monapathi et al., 2017	Diversity	Rivers	South Africa	Physiological tests, Sanger	Ascomycota	Done	-	-

sequencing								
Monapathi et al., 2018	Resistant mechanisms	Rivers	South Africa	Molecular, Sanger sequencing	Ascomycota	Done	Done	-
Clinical studies								
Naglik et al., 2003	virulence	Clinical isolates	United Kingdom	-	Both	-	-	Proteinases
Consolaro et al., 2006	virulence	Clinical isolates	Brazil	Morphology, physiological tests, molecular methods	Ascomycota	-	-	Hyphae formation, biofilm production
Chong et al., 2010	Antifungal susceptibility	Clinical isolates	Australia	DNA fingerprinting and restriction fragment length polymorphism (RFLP) analysis	Basidiomycota	Done	-	-
Kofla et al., 2011	Resistant mechanisms	Hospital	Germany	-	Ascomycota	Done	Done	-
Abrantes et al., 2014	Drug resistance	Hospital	South Africa and Cameroon	Morphology, physiological tests	Ascomycota	Done	-	-
Da Silva-Rocha et al., 2014	Distribution, genotyping and virulence factors	Hospital	Brazil	Morphology, physiological tests, DNA fingerprinting	Ascomycota	-	-	Phospholipase activity, morphogenesis
Leach and Cowen, 2014	Virulence	-	Canada	-	Ascomycota	-	-	High temperature
Dynowska et al., 2014	Antifungal	Hospital	Poland	Morphology, physiological	Ascomycota	Done	-	-

	susceptibility			tests				
Bittinger et al., 2014	Diversity	Hospital	USA	Molecular methods; NGS				
Flowers et al., 2015	Resistance mechanisms	Clinical isolates	USA	-	Ascomycota	Done	Done	-
Kumar et al., 2015	Virulence	Hospital	India	Morphology, physiological tests, Sanger sequencing	Ascomycota			Phospholipase, proteinase and hemolysin activity
Kumar et al., 2016a	Diversity	Hospital	India	Morphology, physiological tests, DNA fingerprinting, Sanger sequencing	Ascomycota	Done	-	
Culakova et al., 2015	Resistant mechanisms		Slovakia	-	Ascomycota	Done	Done	-
Mane et al., 2016	Resistant mechanisms	Hospital	India	Physiological tests, molecular tests	Ascomycota	Done	Done	-
Moges et al., 2016	Antifungal susceptibility	Hospital	Ethiopia	Physiological tests	Ascomycota	Done	-	-
Choi et al., 2016	Gene expression	Clinical isolates	Korea	-	Ascomycota	Done	Done	-
Liu et al., 2016	Gene expression	Hospital	China	-	Ascomycota	Done	Done	-
Nyazika et al., 2016	Antifungal susceptibility	Hospital	Zimbabwe	Physiological tests, molecular tests	Basidiomycota	Done	-	-
Owotade et al., 2016	Antifungal susceptibility	Hospital	South Africa	Morphology , physiological tests	Ascomycota	Done	-	-

Imabayashi et al., 2016	Antifungal susceptibility	Hospital	Japan	Physiological tests, molecular methods; NGS	Ascomycota	-	-	-
Shahrokh et al., 2017	Gene expression	Hospital	Iran	Physiological tests, molecular methods	Ascomycota	Done	-	-
Tasneem et al., 2017	Prevalence, antifungal susceptibility	Hospital	Pakistan	Physiological and molecular methods	Ascomycota	Done	-	-
Mnge et al., 2017	Drug resistance	Hospital	South Africa	Morphology , physiological tests	Ascomycota	Done	-	-
Sherry et al., 2017	virulence	Hospital	Scotland	-	Ascomycota	Done	-	Biofilms
Rocha et al., 2017	Gene expression	Animals	Brazil	-	Ascomycota	Done	Done	-
Canela et al., 2017	Virulence	Hospital	Brazil	Physiological tests , molecular tests	Ascomycota	Done	-	Extracellular enzymes activity
Hampe et al., 2017	Resistant mechanisms	Hospital	Germany	-	Ascomycota	Done	Done	-
Mendes at al., 2018	Antifungal susceptibility	Wild animals, cow's milk with subclinical mastitis and hospital environment	Brazil	Biochemical tests	Both	Done	-	-

Clinical yeast studies have largely been conducted from hospital samples (Kofla et al. (2011), Abrantes et al. (2014), Da Silva-Rocha et al. (2014), Dynowska et al. (2014), Kumar et al. 2015, Nyazika et al. (2016), Owotade et al. (2016), Imabayashi et al. (2016), Shahrokh et al. (2017), Mnge et al. (2017), Canela et al. (2017). Studies from natural aquatic include freshwater (lakes, ponds and rivers; Sláviková and Vadkertiová, 1997, Medeiros et al., 2008, Biedunkiewicz and Baranowska, 2011, Van Wyk et al., 2012, Biedunkiewicz et al., 2013, Brilhante et al., 2016, Brandão et al., 2017, Monapathi et al., 2017), drinking water (Yamaguchi et al., 2007, Pereira et al., 2009, Novak Babič et al., 2016), marine environments (estuaries, coasts, mangrove areas, oceans and the deep sea; Bogusławska-Was and Dabrowski, 2001, Gadanho et al., 2003, Nagahama et al., 2001) and groundwater (Samah et al., 2014, Novak Babič et al., 2016). The presence, diversity and significance of yeasts in such environments has had a limited focus (Arvanitidou et al., 2002).

To understand the state of knowledge with regards to yeasts in freshwater systems a structured review was conducted. Literature that reported on the characteristics, diversity and health implications of aquatic yeasts were surveyed using the following databases: EBSCOhost, Google scholar, Sabinet as well as Science Direct were used to find appropriate citations. Initially, an overall key search using various combinations of relevant words (yeasts, identification, uses, aquatic environments, microbial pollution, yeast infections, antifungal resistance and resistance mechanisms). Only those that simultaneously addressed preselected criteria were selected for further perusal.

2.2. Yeast diversity in freshwater environments: Interplay between physico-chemical and microbiological parameters

The distribution of yeasts species in rivers and lakes is generally similar and comprises species of *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Trichosporon* (Dynowska, 1997, Gadanho and Sampaio, 2004, Medeiros et al., 2008, Van Wyk et al., 2012, Biedunkiewicz and Baranowska, 2011, Brandão et al., 2010:2011:2017, Monapathi et al., 2017). Although, yeasts are common in different aquatic systems, their ecology and implications in the environment is becoming apparent. From these studies, it is evident that the distribution and diversity of yeast species as well as their numbers and metabolic characteristics are influenced by existing environmental conditions (Kutty and Philip, 2008). These consist of abiotic and biotic factors such as physical and chemical characteristics of the river system (Brandão et al., 2011).

In natural environments, the survival of yeasts is maintained by physical and chemical conditions in the ecosystem (Daek, 2006). Most yeasts are mesophilic and grow best at temperatures between 20 and 25°C. Higher temperatures, in the range of 30 and 37 °C are often associated with pathogenic yeasts (Kurtzman et al., 2011). Yeasts prefer a slightly acidic medium with optimum pH between 4.5 and 5.5 (Daek, 2006). Furthermore, yeasts are able to grow aerobically on particular carbon compounds such as alcohols, organic acids and amino acids as their sole energy source (Rodrigues et al., 2006). Daek, (2006) stipulated that increased dissolved oxygen and dissolved organic matter in aquatic environments favours yeast growth. Yeasts can also utilize a wide range of nitrogen compounds as nitrogen sources.

Some nitrogen containing compounds, such as amino acids and ammonia can also be used by yeasts as carbon sources (Dharmadhikari, 2002, Messenguy et al., 2006).

Human activities such agricultural, transportation, energy production, waste disposal and industrial processes affect surface water quality (Chapman et al., 2016) and discharge of pollutant from these activities expose freshwater systems to a variety of organic and inorganic nutrient stress, heavy metals and biological material (Ford, 2000). The impacts of these pollution events results in selection and maintenance of a diversity of yeasts in receiving water body (Jan et al., 2014). However, in water quality assessment studies, there is limited information on the association between yeasts and physical and chemical parameters. In a study conducted by Monapathi et al. (2017), a correlation was seen between yeast levels and Total Dissolved Solids (TDS), nitrates and phosphates. A positive relationship has also been observed between yeasts growth levels with pH, temperature, nitrates and total phosphorus (Jan et al., 2014).

The monitoring of microbial water pollution is based on bacterial faecal indicator bacteria (total coliforms, faecal coliforms, *Escherichia coli*, faecal streptococci: Pereira et al., 2009, Adeleke and Bezuidenhout, 2011). Yeasts as a potential tool for water quality monitoring been overlooked. However, some water quality studies have used yeasts to complement bacterial data e.g. Coliforms and faecal *Streptococcus* counts are used as faecal pollution indicators. Yeasts have complemented these

bacteria as indicators of sewage contamination and recreational water pollution (Hagler, 2006). Opportunistic pathogen, *C. albicans* is present in feces of warm-blooded animals. Moreover, from its association with the human body it can be washed off during bathing (Hagler, 2006). A study by Papadopoulou et al. (2008) has reported on *C. albicans* resistance to chlorine treatment in swimming pools. The resistance makes it an indicator for swimming pool water quality (Sato et al., 1995). Brandão et al. (2010) correlated yeasts that grow at 37°C with the faecal coliform group as complementary microbial indicators in polluted aquatic environments containing high organic loads from human origin. Incubations at 37°C was a selective temperature for growth of opportunistic yeast pathogens. The quick response of yeasts to organic contamination makes it a valuable indicator of nutrient enrichment in aquatic environments.

Studies have demonstrated a correlation between yeast levels and physico-chemical parameters in contaminated water. From a study conducted by Sláviková and Vadkertiová (1997) in the Danube River, high concentrations of yeasts (up to 21×10^3 CFU/L) were found in water samples. At the time the river had eutrophication problems characterized by increased amounts of nitrogen and phosphorus. Simard (1971) studied yeast association at sewage pollution and urbanization interphases with relevance to physico-chemical parameters and yeasts levels. The author found that Biological Oxygen Demand (BOD) and Dissolved oxygen (DO) had an effect on yeast numbers that ranged from 4800-10900 CFU/L. In a study conducted by Monapathi et al. (2017), nitrates, phosphates and temperature conditions indicated that the river systems provided an ecological habitat that could maintain growth of relatively high levels (up to 2,573 CFU/L) of yeasts.

Yeast species present in the environment can also be linked to the degree and type of pollution that has occurred (Hagler and Mendonca-Hagler, 1981). Early studies have established that in clean water there are large numbers of non-fermentative yeast species. On the other hand, polluted waters are dominated by a denser population of mostly fermentative yeasts (Hagler and Ahearn, 1987, Hagler, 2006). *S. cerevisiae* is uncommon to pristine aquatic habitats. Its presence in large numbers in water systems could thus be an indication of pollution (Hagler, 2006). Studies conducted in freshwater environments have concluded that *C. albicans*, *Candida famata*, *Cryptococcus laurentii*, *Pichia guilliermondi*, *Rhodotorula glutinis*, *R. mucilaginosa*, *Trichosporon beigelii* and *Wickerhamomyces anomalus* are associated with wastewater pollution (Dynowska et al., 1997, Lahav et al., 2002, Hagler, 2006, Nagahama, 2006). These examples and what we know at this stage have all demonstrated that yeast levels and diversity could potentially be informative to complement but also as alternatives to faecal indicator bacteria.

2.3. Yeasts as opportunistic pathogens

Globally, the number of deaths from yeast infections is comparable to those caused to malaria and tuberculosis (Bongomin et al., 2017). Yeast infections such as thrush, athlete's foot and ring worm, are superficial and affect the skin or mucous membranes. Life threatening infections invade blood stream and disseminate to internal organs and cause meningitis and candidiasis (Gould, 2012, Whaley et al., 2016). Immunocompromised individuals are at higher risk for yeast infections. These are mostly patients in therapeutic technology such organ transplant, anticancer

therapies and certain disease conditions such as malignancy and HIV (Pincus et al., 2007, Richardson and Lass-Florl, 2008).

Globally, the prevalence of yeast infections is increasing (Paramythiotou et al., 2014). Most infections are frequently caused by pathogens from the genera *Candida* and *Cryptococcus* (Tlamcani and Er-ram, 2013). These are known to cause candidiasis and cryptococcosis. Thus the presence of these two genera in water sources, poses health risks to communities that use water for domestic and agricultural purposes as well as activities where direct exposure is common such as recreation and religious cleansing or baptism (Zenani and Mistri, 2005). Direct contact with water polluted with these pathogenic yeasts could cause diseases/infections in healthy individuals (Monapathi et al., 2017). This is a public and health concern and needs more research to highlight this aspect but also to generate sufficient data to evaluate if policy changes are required for including yeasts in water quality guidelines.

Virulence is the ability of a pathogen to cause damage to host tissues (Casadevall, 2007). Various virulence factors are associated with pathogenic/opportunistic pathogenic yeasts. Detailed knowledge about these factors in yeasts is limited (Yu et al., 2017). Attributes such as high temperature, morphogenesis, secretion of extracellular enzymes, capsule formation and formation of biofilms are all considered virulence factors (Mayer et al. 2013). The following sections deal with these factors.

2.3.1. High temperature and morphogenesis

The ability to grow at elevated temperatures, particularly human body temperature (37°C) is considered a specific virulence feature (Leach and Cowen, 2014). Yeasts, particularly *Candida* spp., were isolated from aquatic environments and demonstrated the ability to grow at 37°C (Brandão et al., 2010). Such elevated temperatures facilitate the morphogenetic transition and this facilitates their multiplication inside the host (O'Meara and Cowen, 2014). The thermal adaptation and heat shock at 37°C had been mostly studied in *C. albicans* and *C. dubliniensis* (Saville et al., 2003, Khan et al. 2010, Pereira, 2015). At 37°C pathogenic yeast *C. neoformans* survive better than their non-pathogenic counterparts (Hogan et al., 1996).

Many *Candida* spp. form filamentous pseudohyphae and hyphae in tissues. Hyphal forms facilitate their multiplication within the host at higher temperature. *Cryptococcus neoformans* becomes coated with a capsule (Rappleye and Goldman 2006). These morphogenic virulence mechanisms facilitate tissue damage and invasion (Khan et al., 2010).

2.3.2 Extracellular enzyme production

Production of extracellular enzymes such as proteinases, phospholipases, lipases and keratinases had been implicated as virulence factors in yeasts. These enzymes enable nutrient uptake, tissue invasion, adherence, and dissemination inside the host (Khan et al., 2010).

Proteinases are the most studied enzymes and are regarded as the major factors of virulence that degrade the tissue barriers and obtain nutrition at the infection site of the host (Liu et al., 2011). The secretion of aspartic proteases (Asp), encoded by a gene family with 10 members has been documented as a virulence-associated trait in *C. albicans* (Pietrella et al., 2010). Phospholipases when secreted hydrolyze ester linkages of glycopospholipids and hence impart tissue invasiveness to *Candida* cells. Keratinases damage the keratinous layer in epidermis of the host. The ability of pathogenic yeasts to degrade keratin is considered a major virulence attribute (Khan et al. 2010, Achterman and White, 2012). Monapathi et al. (2017) isolated various *Candida* spp. from aquatic systems that were able to produce extracellular enzymes.

2.3.3 Capsules and proteins

The presence of a polysaccharide capsule in yeast species such as *C. neoformans* may also play an important role in the virulence capabilities of this species. The capsule surrounds the cell wall and has multiple virulence effects on the host immune system (Bose et al., 2003). Production of capsules in *C. neoformans* is a morphogenic virulence factor triggered by high human host temperature (Khan et al., 2010). Capsules activate immune system cell apoptosis and this inhibits normal human immune response such as the production of antibodies, presentation of antigen, migration of leukocytes and complement activity (Araújo et al., 2017).

Some yeasts have specialized sets of proteins (adhesins) which enable them to adhere to other yeast species, abiotic features and host cells. Two most frequently encountered species in hospital-acquired infections, *C. glabrata* and *C. albicans* adhere to host epithelial cells during infection (Calderone and Fonzi, 2001, Desai et

al., 2011). Other forms of proteins that facilitate virulence in yeasts are invasins. These mediate binding onto host ligands thereby triggering engulfment of the yeast cell into the host cell (Mayer et al., 2013). Studies by Almeida et al. (2008) and Liu et al. (2011) on *Candida albicans* have shown invasins as vital virulence factors. In the study of Monapathi et al. (2017), various *Candida* spp. (including *C. albicans* and *C. glabrata*) were isolated from aquatic systems.

2.3.4 Biofilm formation

Biofilms are communities of microorganisms that exist as organized structures attached to some inanimate surfaces or tissues and are found in a matrix of exopolymeric materials (Khan et al., 2010). Production of biofilms by some yeasts especially *Candida* species on catheters, endotracheal tubes, pacemakers and other prosthetic devices has contributed in nosocomial infections (Douglas, 2003, Ramage et al., 2006). *Candida albicans* is the most studied yeast among the biofilm formers. Other yeast species such as *C. glabrata*, *Candida tropicalis* and *C. parapsilosis* and *Cryptococcus neoformans* have also been implicated in biofilm-associated infections (Ramage et al., 2006, Negri et al., 2011, Martinez and Casadevall, 2015, d'Enfert and Janbon, 2016). Monapathi et al. (2017) identified all of these mentioned species amongst the yeasts isolated from aquatic systems and had the ability to grow at 37°C.

2.4. Antifungal agents used to treat yeast infections

Infections such as thrush, athlete's foot, ring-worm, candidiasis, jock itch and meningitis are caused by various yeast species (Gould, 2012). Antifungal drugs are available to treat such infections. These drugs can be classified into 4 different classes based on their mechanisms of action: (1) alteration of membrane function (2) inhibition of DNA or RNA synthesis (3) inhibition of ergosterol biosynthesis (4) inhibition (Perea and Patterson, 2002). Some of the yeasts studies that determined antifungal resistance in yeasts are emphasized in Table 1. A global increase in HIV pandemic has resulted an increase in opportunistic yeast infections in HIV infected people from *Candida* and *Cryptococcus* spp. (Morschhäuser, 2016). Antifungal agents, such as fluconazole, were also introduced as prophylactic option in HIV treatment (Morschhäuser, 2016).

2.4.1. Azoles

Azoles inhibit the yeast cytochrome P450 enzyme, 14 α -demethylase, during ergosterol biosynthesis. Ergosterol is the main stabilising component in the yeast cell membranes. Inhibition of the ergosterol synthesis pathway leads to cell membrane stress and growth inhibition (Sanglard, 2016). The antifungal azole drug class is composed of imidazoles (clotrimazole, ketoconazole, miconazole) and triazoles. Triazoles are categorised as first-generation azole drugs and include fluconazole and itraconazole. These became available in the 1990s. Newer agents, second-generation of azole drugs include voriconazole, posaconazole, and isavuconazole which became available beginning in the 2000s (Nett and Andes, 2015). During

systemic administration, imidazoles exhibit significant toxicity. However, imidazole drugs are also available as topical preparations for the treatment of skin yeast infections (Pappas et al., 2016). Triazoles have a broader spectrum of antifungal activity and an improved safety profile than imidazoles (Maertens, 2004).

Fluconazole is the most widely prescribed antifungal in South Africa (Truter and Graz, 2015). It is mostly preferred because of its high oral availability and tolerability by patients (Sanglard, 2016). Fluconazole is active against medically important yeasts species, namely *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *C. dubliniensis* and *Cryptococcus neoformans* (Pfaller et al., 2004). It is used to treat mucosal and systemic candidiasis, cryptococcosis, and is used as a prophylaxis for candidiasis (Nett and Andes, 2015).

Itraconazole has a good activity against *Candida* spp. (such as *C. glabrata* and *C. krusei*) and *Cryptococcus neoformans* (Pfaller et al., 2001). It has been used in the treatment of numerous mycoses including mucosal, oropharyngeal, oesophageal, vaginal candidiasis and cryptococcosis (Smith et al., 1991, Pappas et al. 2016, Nett and Andes, 2015). It also prevents invasive yeast infection in hematologic malignancy or autologous bone marrow transplantation patients (Menichetti et al., 1999, Nucci et al., 2000).

Voriconazole and posaconazole have high antifungal activity against *Candida* species (including fluconazole resistant *C. krusei*, *C. glabrata*, *C. albicans* and *Cryptococcus neoformans* (Nagappan and Deresinski, 2007). Both antifungal agents are approved for the treatment of mucosal, esophageal, invasive candidiasis and prophylaxis for invasive yeast infection. Isavuconazole is active against *Candida* spp. including *C. glabrata* and *C. krusei* (Nett and Andes, 2015).

Antimycotic agents are also used in agriculture for plant protection (Jampilek, 2016, Dalhoff, 2017). These agents include antifungal derivatives, azoles such as chemically synthesised benzimidazoles. Azoles are the most used agents because they are inexpensive and have a broad spectrum of antifungal activity. However, the broad spectrum treatment and exposure of yeasts to azoles has led to resistance problems with consequences on human health (Snelders et al., 2009).

Plants and fruits serve as natural habitats for many potentially human pathogenic types of yeast such as *Cryptococcus* (Fleet, 2003). During irrigation and rain episodes these azole resistant yeasts from the agricultural settings are transported from the plants and fruit into receiving bodies.

2.4.2. Polyenes

Polyenes bind to ergosterol in the cell plasma membrane damaging membrane barrier function (Scorzoni et al., 2017). These antifungal agents form pores on the plasma membrane and this results in the leakage of cellular components, interference with ion and electrical gradients and ultimately, cell death.

Amphotericin B and nystatin are the two polyene derivatives commonly used (Sanglard et al., 2009, Tscherner et al., 2011, Jensen, 2016) and are active against *Cryptococcus* spp. and most *Candida* spp. excluding *Candida lusitanae* (Diekema et al., 2003, Pfaller et al., 2003). They are thus used in the treatment of candidemia and invasive candidiasis (Gallis et al., 1990, Lyu et al., 2016).

2.4.3. Echinocandins

Echinocandins inhibit the catalytic subunit of the 1, 3- β -D-glucan synthases. This is the enzyme responsible for the synthesis of the yeast cell wall component, polysaccharide β -1,3-glucan (Arendrup and Perlin, 2014). In the presence of echinocandins, the integrity and structural organization of the cell wall is disrupted leading to fungicidal action (Douglas, 2001, Jensen, 2016). Echinocandins agents are active against many *Candida* spp. including *C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. tropicalis*, and *C. krusei*. This class of antifungal agents is used to prevent invasive yeast infections and treatment of candidiasis (Villanueva et al., 2002, de Wet et al., 2005, Reboli et al., 2007).

2.4.4. Nucleoside analogues

Nucleoside analogues simulate physiological nucleosides and exert their cytotoxic effects by getting incorporated into DNA during normal DNA synthesis. This results in inhibition and chain termination (Ewald et al., 2008). Some of the analogues destabilise the deoxynucleotide pool balance by inhibiting key enzymes involved in generation of purine and pyrimidine nucleotides. This ultimately interferes with DNA and RNA synthesis. Flucytosine is an example of a nucleoside analogue (Galmarini et al., 2002) and has antifungal activity against many *Candida* spp. such as *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. lusitaniae* and *Cryptococcus* spp. (Nett and Andes, 2015). It is used as first-line therapy for the treatment of cryptococcal meningitis and can also be used to treat *Candida cystitis* (Vermes et al., 2000, Pfaller et al., 2005).

2.5. Antifungal resistance mechanisms

All these classes of antifungal agents are used against a variety of pathogenic yeast infections and have proven to be effective. However, resistance which is caused by continuous exposure of yeasts to antifungal agents has been reported (Morschhäuser, 2016). For example in the early 1990, fluconazole was the antifungal of choice in treating oro-oesophageal candidiasis. However, in the years following this, fluconazole resistance was reported in 41% of patients (Canuto and Rodero, 2002). In a clinical study conducted by Mnge et al. (2017), *C. albicans* resistance to fluconazole was 4.6%, voriconazole resistance (2.8%) and flucytosine resistance (3.7%). Mendes et al. (2018) isolated different yeast species from wild animals,

cow's milk with subclinical mastitis and hospital environment and performed antifungal susceptibility tests. From 89 yeasts, 20.9% were resistant to fluconazole, 12.4% to amphotericin B and 3.4% to voriconazole. Yeasts isolated from the hospital environment showed the highest resistance (13.5%) to fluconazole.

There has been a concerted effort to monitoring and report on resistance development among clinical isolates. However, environmental yeast isolates has generally been ignored. The presence of antifungal agents in any environment affects the diversity and selection of antifungal resistant pathogens/opportunistic pathogens. Up to now limited studies were conducted on antifungal resistance among aquatic yeasts (Medeiros et al., 2008, Brandão et al., 2010, Brilhante et al., 2015:2016, Monapathi et al., 2017:2018). Medeiros et al. 2008 performed an antifungal resistance study on yeast isolates from lakes and rivers. Most of the yeasts isolates were resistant to ketoconazole and terbinafine. Fifty percent of the yeast isolates were resistant to itraconazole. Antifungal susceptibility results from a study by Brandao et al. 2010 in freshwater lakes showed yeast resistance to amphotericin B (21.7%), followed by itraconazole (20%) and then fluconazole (2.8%). In Monapathi et al. (2017), isolated yeasts were 100% resistant to miconazole and flucytosine. Furthermore, 88.5 and 92.5% fluconazole resistance was observed for Mooi and Harts river, respectively..

Antifungal resistance mechanisms have been studied at a molecular level (Sanglard, 2016). An understanding of antifungal resistance at molecular level is vital when developing approaches against such resistance. It is also important in validating the design of newer antifungals and target-based molecular approaches. However the

molecular basis focus had been, until recently, only on clinical isolates (Culakova et al., 2015, Bosco-Borgeat et al., 2016, Choi et al., 2016, Liu et al., 2016, Hampe et al., 2017, Rocha et al., 2017). These resistance mechanisms in pathogenic yeasts species such as *Candida albicans*, *C. glabrata*, *C. tropicalis* and *Cryptococcus neoformans* are centered on transport alterations, target alteration and use of differential pathways (Cowen et al., 2015, Sanglard et al., 2016). Recent studies on yeast resistance mechanism among aquatic yeasts (Brilhante et al., 2016, Monapathi et al., 2018) have demonstrated that mostly similar mechanisms were present in environmentally isolated *Candida* sp. when compared to clinical isolates.

2.5.1 Transport alterations

Resistance by enhanced efflux multidrug transporters is the most ubiquitous resistance mechanisms (Blanco et al 2016). Antifungal resistant isolates with their efflux pumps transport antifungal drugs out of the cell. With reduced accumulation of therapeutic drug in the cells, resistant isolates survive (Prasad and Rawal, 2014). Multidrug transporters are ATP- binding cassette (ABC) transporters (encoded by *Candida* drug resistant, *CDR1* and *CDR2* genes) and major facilitator (MF) drug pumps (encoded by multidrug resistance, *MDR1* genes and fluconazole resistance, *FLU1* genes) (Cowen et al., 2015, Sanglard et al., 2016). Clinical and environmental studies have reported on antifungal resistance as a result of enhanced efflux pumps in pathogenic yeast (Mane et al., 2016, Brilhante et al., 2016, Choi et al., 2016, Rocha et al., 2017).

2.5.2 Target alteration

Target alterations are known resistance mechanisms for azoles. Resistance mediated by alterations on the target enzyme cytochrome P-450 lanosterol 14 α -demethylase (encoded by *ERG11* gene) as a result of a mutation and/or overexpression has been widely documented (Cowen et al., 2015, Morschhäuser, 2016, Sanglard et al., 2016). *ERG11* is highly permissive to structural changes resulting from amino acid substitutions. Antifungal resistance mediated by changes in target enzymes encoded by *ERG11* gene has been reported in pathogenic yeasts (Culakova et al., 2015, Flowers et al. 2015, Choi et al., 2016, Rocha et al., 2017, Monapathi et al., 2018).

2.5.3 Use of differential pathways

Altered sterol biosynthetic pathway results in the replacement of ergosterol by other sterols in the cytoplasmic membrane (Li et al., 2018). Azole resistance in *C. albicans* mainly comprises the inactivation of the enzyme sterol $\Delta^{5,6}$ desaturase (encoded by *ERG3*). Erg3 catalyzes the introduction of a carbon-carbon double bond in the substrate molecules, one of the final steps in the ergosterol biosynthetic pathway. Moreover, it converts the nontoxic 14 α -methylfecosterol that accumulate during azole treatment into toxic sterol 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol. The inactivation or deletion of the *ERG3* gene is important in preventing the synthesis of such toxic substances (Morio et al., 2012, Vale-Silva et al., 2012, Cowen et al., 2015, Whaley et al., 2016).

2.6. Route of antifungals into water systems

Sub-therapeutic levels of antifungal agents constantly land into aquatic environments (Singer et al., 2016, Meade 2017). This is linked to prevailing therapy regimes (infection control or prophylactic treatment), disposal routes and waste (wastewater) treatment options. In the case of the prophylactic use of fluconazole, resistance of yeast from patients has increase dramatically (Morschhäuser 2002, Abrantes et al., 2014).

Of the 36.7 million people globally were living with HIV, only 20.9 million have access to antiretroviral therapy (UNAIDS, 2017). In South Africa, 3,9 million people (of the 7.06 million) are on antiretroviral therapy (Statistics S.A, 2017). Antiretroviral therapy includes the prophylactic and infection control treatment using fluconazole. The drug is administered at a dosage of 400–800 mg per day (Longley et al., 2008). However, fluconazole is partially metabolized by the human body and a large proportion (up to 75%) of the dose is excreted in urine. It finds its way into the sewage system, subsequently into WWTP and then reaches the receiving environment (Kim et al., 2007, Kahle et al., 2008, Kim et al., 2009). Fluconazole concentrations measured in China WWTPs were 22-170 ng/L and 50-139 ng/L in the influent and final effluent, respectively (Peng et al., 2012). These concentrations fall within the same ranges with the studies conducted in WWTPs by Kahle et al. (2008) in Switzerland and Lindberg et al. (2010) in Sweden. The authors measured fluconazole concentrations between 10 and 110 ng/L and 40 and 140ng/L, respectively. Fluconazole concentration ranges from surface water in Korea measured from not detected (ND)

to 111ng/L (Kim et al., 2009). These aforementioned studies have largely linked the high concentrations of fluconazole to clinical use.

WWTPs that work efficiently, only partially remove pharmaceutical products (Kümmerer, 2009). Kahle et al. (2008) conducted a study on azole antifungals in wastewater and surface water. These authors demonstrated that fluconazole is not removed by conventional wastewater treatment processes. Its concentrations were averagely similar in both treated wastewater and untreated wastewater. Poorly treated wastewater may also contain large amounts of other antimicrobial agents that could decant into the rivers (Yang et al., 2014). DWAF. (2006) conducted a national survey on 51 treatment plants in eight provinces in South Africa. The authors demonstrated that most treatment plants are faced with challenges such as small scale operation, poorly maintained or old infrastructure as well as shortage of trained, skilled and experienced process controllers as well as mechanical/electrical maintenance staff. Analysis of the 2012/13 Green Water Services has indicated that many municipal waste treatment plants (30.1%) did not meet regulatory expectations in terms of compliance and best practice requirements (DWAF, 2013a). In the South African context, WWTPs could thus be regarded as major contributors to opportunistic pathogenic yeast species into environmental water systems.

Agricultural activities also play a major role in the antifungals in our water systems. Azoles are used to protect particularly grain crops against various fungal diseases of grains (Jampilek, 2016, Dalhoff, 2017). They are also sprayed every year over the vegetables and fruits to control mildews, rust, and other diseases (Azevedo et al., 2015). The recent use rates for triazole according to manufacturers' instruction are

below/or around 100 g/ha of plant surface. This corresponds to 10 mg of azoles applied to 1 m² of the plant surface (Azevedo et al., 2015). During rainy seasons, several applications are sometimes necessary (Hof, 2001). Chemically synthesized antimycotic agents; ithiocarbamates, phthalimides, fentins and benzimidazoles were used in quantities ranging from 1.5 to up to 3 kg per hectare (Morton and Staub, 2008). Runoff of these fungicides from agricultural soil will also land in the water and impact on the water system.

2.7. Public health concern of finding pathogenic yeasts in environmental water

An increasing number of immunosuppressed patients have resulted in elevated frequent diagnoses of invasive yeasts infections (Miceli et al., 2011). The prevalence rate of HIV in South Africa is estimated at 12.6% (Statistics S.A, 2017) and these patients are at risk of contracting an invasive yeast infections. Most studies have focused on resistance in clinical isolates (Abrantes et al., 2014, Moges et al., 2016, Mnge et al., 2017). Some studies have revealed antifungal resistance in environmental yeasts (Medeiros et al. 2008, Monapathi et al., 2017). The incidence of yeast species resistant to commonly used antifungal agents shows that these microorganisms could pose a health risk to water users (Brandão et al., 2010). Surface water in the NWP is used for household, recreational and religious purposes that include direct exposure to individuals (NWP-SoER, 2014).

For aquatic yeasts, contact transmission is normally the route of infection (Tong, 2017). Thus the presence of pathogenic yeasts in our freshwater environments could

affect the general population but could have devastating effects for the immunocompromised section of communities. According to the ARTEMIS Global Antifungal Surveillance Program, *Candida albicans* is the major (63-70%) cause of invasive yeast infections from yeasts (Pfaller et al., 2007). It causes candidemia, a leading cause of morbidity and mortality in the health care settings (Horn et al., 2009). *Candida glabrata* is the second most common (44%), followed *Candida tropicalis* (6%), and *Candida parapsilosis* (5%) (Pfaller et al., 2007). However, this order is determined by geographical and institutional differences (Miceli et al., 2011). *Candida* spp. infections range from superficial candidiasis infections to systemic or invasive candidiasis. Superficial infections can occur in the skin and mucous membranes. Systemic or invasive candidiasis includes disseminated candidiasis, candidemia, endocarditis and meningitis and these infections have a mortality rate of 40-50% (De Rosa et al., 2009, Saranya et al., 2014).

Trichosporon spp. cause invasive trichosporonosis, a rare but frequently fatal mycosis in immunocompromised patients (Girmenia et al., 2005). The yeast species are documented to have caused infections such as fungemia with cutaneous dissemination, endocarditis and chronic dissemination (Colombo et al., 2011). The genus *Rhodotorula* spp. (*R. mucilaginosa*, *R. glutinis*, and *R. minuta*) are known to cause disease in humans (Larone, 1995). Fungemia caused by *Rhodotorula* spp. have been associated with central catheters in patients with haematologic malignancies (Zaas et al., 2003). *Cryptococcus* spp. (*Cryptococcus laurentii* and *Cryptococcus albidus*) are responsible for 80% of the cases of cryptococcosis (Leite et al., 2012). *S. cerevisiae* can cause invasive diseases in immunocompromised

patients (Pillai et al., 2014, Popiel et al., 2015). All these yeast species have been isolated from surface water elsewhere and in South Africa (Van Wyk et al., 2012, Monapathi et al., 2017). There is lack of studies that link these isolates/strains from the environment to those from clinical settings.

Rivers and lakes are the sources for our drinking water. The water is purified through various processes and are disinfected before they are distributed to consumers. If water is inefficiently treated, yeast from the water source could end in drinking water (De Toni, 2011). Consumption from yeast contaminated water has not caused acute diseases in healthy individuals (Hageskal et al., 2009). However, there is a risk of superficial or localised infection in these healthy individuals and more severe and invasive infection in immuno-compromised persons.

2.8. Quantitative risk assessments

Most of the pathogenic yeasts isolated until now have been from clinical samples where known infections occurred (Agarwal et al., 2011, Borman et al., 2012). Finding similar species in environmental water is thus really cumbersome. From clinical tests, for microorganisms to cause an infection, the number of colony forming units CFU/ml in bloodstream should be defined. In bloodstream infection (BIS), *Candida* CFU/ml in the first 50% positive blood culture had <1 CFU/ml of circulating organisms (Pfeiffer et al., 2011). For candidemia, classified as high-grade and low-grade candidemia, 25 colony-forming units or more per 10 ml and 10 CFU or fewer per 10 ml of blood respectively were defined (Telenti et al., 1991). According to

Perlin and Wiederhold. (2017), a low initial concentration (often <10 colony-forming units [CFU]/mL) of the pathogen within the collected specimen can grow to cause an infection. This suggests that low levels of yeasts in direct exposure to human could cause an infection. Similar quantitative risk data for environmental exposures are not available and there is a need to generate this.

2.9. Conclusion

The number of peer-reviewed articles about yeast diversity in water has increased and some have originated in South Africa. The present review largely focused on freshwater environments. Most of the studies on yeasts in aquatic environments address water pollution aspects. Yet, bacterial indicator species are mainly the microbes that are used in water quality assessments. Declining water quality is a global concern and in these systems the chemical and physical conditions are such that yeast species could survive. Some of these are known pathogenic or opportunistic pathogens. Future studies are needed to generate data to determine whether it is necessary to include yeasts in water quality guidelines. This may be necessary, if one considers the large sections of populations in developing countries that are immunocompromised, particularly those living with HIV. Studies on health implications have mostly been addressed on pathogenic clinical isolates. This creates a gap in research as similar isolates have been isolated from aquatic environments. The same resistance patterns to antifungal agents and resistance mechanisms associated with clinical isolates were also found to exist among environmental isolates. Molecular methods to study antifungal resistance mechanisms should be extended to environmental isolates. Direct exposure to polluted water is a health threat. Studies in the clinical settings have shown that to

cause an infection, yeast level as low 1 CFU/ml is sufficient. Similar data for environmental water levels are needed. More studies are needed in South Africa and globally to address the possible implications of antifungal resistant pathogenic yeasts in water.

CHAPTER 3

Physico-chemical levels and culturable yeast diversity in surface water: A consequence of pollution

3.1 Introduction

Human activities in and around aquatic ecosystems have effects on these environments. When these activities are associated with industries, mines, WWTPs and agriculture a range of organic and inorganic pollutants, heavy metals and biological materials may be discharged into the riversystems. The quality of the water will inevitably deteriorate affecting aquatic life (Ford, 2000, Ansari and Metondkar, 2014, Gupta et al., 2017). Since the use of surface and subsurface water for various purposes are directed by physico-chemical and biological quality of the water, pollution could reduce the water use prospects (Uddin et al., 2014). Environmental pollutants affect physico-chemical parameters and the microbial density present in the water system (Traoré et al., 2016). Analyses of water properties such pH, dissolved oxygen (DO), chemical oxygen demand (COD) and nutrients are used to determine water quality in accordance with well established standards (Enderlein et al., 1997). It is thus imperative that water resources are routinely monitored to ensure environmental sustainability for optimal water use and resource protection (DWAF, 2009). In South Africa, Department of Water Affairs and Forestry (DWAF, 1996a:1996b) has established Target Water Quality Ranges (TWQR) for several uses to guide decision makers. Recently, the water quality objectives for various river systems were introduced (DWS, 2018). This is to guide

the minimum levels/standards of various parameters of effluent from water users and runoff that would not cause a severe impact on water quality.

Microbial studies that monitor surface water pollution mostly measure bacteria (total coliforms, faecal coliforms, *Escherichia coli*, faecal *streptococci*, and *enterococci*) (Pereira, 2009, Adeleke and Bezuidenhout, 2011). However, Steven et al., (2003) has proposed the use of other groups of microbes as water quality indicators. One such group is the yeasts. Some studies have reported that high yeast levels could be an indication of contaminated water (Medeiros et al., 2008, Van Wyk et al., 2012, Monapathi et al., 2017). Certain types of yeasts are also regarded water pollution indicators. A rapid response of yeasts to organic contamination makes some yeasts important indicators of nutrient enrichment since these convert easily accessible carbon sources into energy for reproduction. *S. cerevisiae* strains indicate contamination of the water with bakery or brewery effluents (Brandão et al., 2010). *Candida krusei*, *C. guilliermondii* and *C. tropicalis*, on the other hand, are associated with faecal contamination of water by warm-blooded animals. Globally, several studies have reported on yeasts as a complement to faecal indicator bacteria counts in monitoring water quality (Hagler, 2006, Medeiros et al., 2008, Brandão et al., 2010).

Some of the pathogenic species isolated from freshwater environments include *Candida albicans*, *Candida parapsilosis*, *C. krusei*, *C. guilliermondii*, *Candida glabrata* and *C. tropicalis* (Brandão et al., 2010:2011:2017, Van Wyk et al., 2012, Medeiros et al., 2008:2012, Monapathi et al., 2017). They can cause various diseases from superficial mucosal infections to life-threatening systemic disorders in

especially immunocompromised people (Ravikumar et al., 2015). These are mostly patients with cancer, organ transplants and HIV patients (Pincus et al., 2007, Richardson and Lass-Flörl, 2008). Increased numbers of immunocompromised people has resulted in increased prevalence of local and systemic infections caused by pathogenic yeasts species (Puebla, 2012).

Antifungal drugs are used to treat these yeast infections. However, yeast species have developed resistance to several of the clinically used agents (Vandeputte et al., 2012). Resistance may result from using selective therapies with inadequate doses or continuous exposure and prophylactic use in human and animals (Galle and Gianinni, 2009, Abrantes et al 2014). Sub-therapeutic levels of antifungal agents are constantly leaked into the environment from ineffective wastewater treatment plants (WWTPs), animal and agricultural use (Mateo et al., 2013, Singer et al., 2016). Moreover, WWTPs and sewage sludge harbour pathogenic yeasts (Dumontet et al., 2001). The release of such into aquatic ecosystems escalates resistance through continuous exposure to antifungal agents. Pathogenic yeast resistance to commonly used antifungal drugs has thus been observed among environmental isolates (Brandão et al., 2010, Medeiros et al., 2012, Monapathi et al., 2017).

From direct contact when using surface water for irrigation, recreational and religious purposes, people are at a risk of infection with yeasts. This is a public health concern in South Africa and particularly NWP where the HIV prevalence rate is approximately 13% (Shisana et al., 2014, Statistics S.A, 2017). The present study aimed to determine pollution from human activities using physico-chemical parameters and yeast levels and how these correlate in four selected river systems in the NWP. The

study addressed antifungal resistance from isolated opportunistic pathogens and possible health implications due to direct contact with the polluted water.

3.2. Experimental section

3.2.1 Study area

3.2.1.1 Mooi River

The Mooi River catchment has a total area of 1800km² and is situated in the western section in Gauteng Province and the eastern North West Province of South Africa. There are three sub-catchment - Mooi River-loop, Wonderfonteinspruit and Loopspruit (Van Der Walt et al., 2002). Local municipalities (Carletonville and JB Marks - Potchefstroom) use the water for domestic drinking water production. There are also mining, farming and production industries that are dependent on the water (Van Aardt and Erdman, 2004). The catchment thus experiences water pollution from urbanization, agricultural and mining activities (DWAF, 2007, Barnard et al., 2013, Venter et al., 2013). There is a serious public health threat to people living in informal settlements along the river where there is no formal water since they use stream-water as the main water source (Winde, 2010).

3.2.1.2 Schoonspruit River

The Schoonspruit River is located in the Middle Vaal WMA. It originates from dolomitic eyes above Ventersdorp and flows through Klerksdorp and Orkney and finally runs into the Vaal River (DWAF, 2012b). Water from the catchment is used for drinking water production, irrigation, mining and livestock farming. The river thus supplies the local municipality with its urban water requirements (DWAF, 2009).

However, runoff and return flows from urban and industrial activities as well diamond digging effluents pollute the river system (DWAF, 2004). The Schoonspruit River is a tributary of the Vaal River, pollution from it also negatively affect water quality in the lower reaches of the Vaal River (Van Vuuren, 2008).

3.2.1.3 Crocodile (West) and Marico River

The Crocodile (West) and Groot Marico River catchment is spread across the three provinces (Gauteng, North West and Limpopo Provinces. These two rivers form the south-western part of the Limpopo River basin (DWS, 2017). Crocodile West River catchment is the largest (29 349 km²) and Marico River catchment the smaller (12 049 km²). Water from the Crocodile West River is used for agriculture, industry, mining and urban drinking water production (DWAF, 2012a). Pollution in the catchment results from irrigation return flow, poorly treated sewage effluent, industrial and mining activities (DWAF, 2009). Land use in Marico catchments is dominated by agriculture and some urbanization. The main pollution contributors are thus agriculture, invasive alien vegetation, limited mining discharges and return flows from urbanisation (DWAF, 2007).

3.2.2 Sampling

A Garmin Nüvi 1310 (Garmin, US) global positioning system (GPS waypoints) was used to locate the water sampling sites. These are shown in Figure 1. There were sampling periods, which represent the wet-warm, and dry-cold seasons. Two sampling techniques were employed using 1L sterile bottles aseptically. The direct sampling method was employed at sampling sites where water samples could be

collected close to the river bank. Dip sampling (using a rope) technique was used where direct access to the surface water systems was limited.

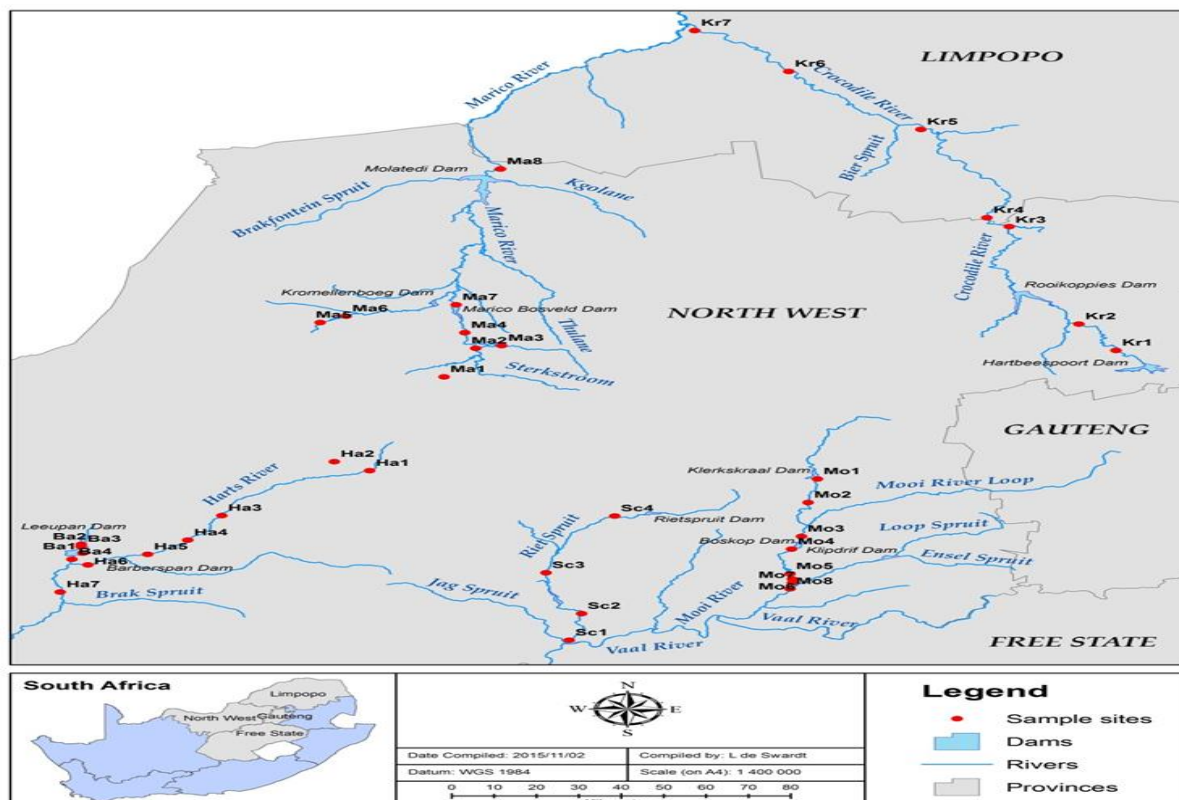


Figure 1. Map showing selected river systems in the North West Province (Mo= Mooi River; Ma=Marico River; Sc= Schoonspruit River; Kr= Crocodile River).

3.2.3 Physico-chemical parameters analyses

Temperature, pH, DO and total dissolved solids (TDS) were determined *in situ* using a multi 350 multi parameter probe (Merck, Germany). Water samples were transported to the laboratory in cooler boxes and on ice. Samples were analyzed within 8 hrs. The laboratory analysis of nitrates (Method 10206), phosphates (Method 8048) and COD (Method 8000) were performed using the Hach Lange DR 2800 system and reagents (Hach Company, 2007).

3.2.4 Yeast isolation, enumeration, biochemical and molecular identification

3.2.4.1 Yeast isolation and enumeration

The water samples were analyzed by membrane filtration to determine the presence of yeasts in water using the method described by Van Wyk et al. (2012).). One hundred milliliters of water samples were filtered in duplicates through 0.45µm HA membranes filters (Whatman®). Membranes were placed on to yeast-malt-extract (YM) plates (YM) : (10g/L glucose; 3 g/ L malt extract; 3 g/L yeast extract; 5 g/L peptone and 15 g/L agar) (Wickerham, 1951), supplemented with 100ppm chloramphenicol. Incubation was done at room temperature and at 37°C for 24 hrs. The formation of yeast colonies was examined daily until day 5 when the colonies were counted. Isolates from the plates grown at 37°C were purified by sub-culturing on YM agar (Wickerham, 1951).

3.2.4.2 Biochemical identification

Diazonium blue B (DBB) (Sigma-Aldrich, Germany) was used to distinguish between ascomycetous and basidiomycetous yeasts. Yeast cultures were grown on YM agar plates and incubated at 37°C for 14-21 days (Kurtzman and Fell, 1998). One to two drops of freshly prepared chilled DBB were applied onto surface of each colony. A positive reaction of dark red to violet red colour within 2 minutes at room temperature showed that that the yeast belongs to basidiomycetes. No colour change signifies a negative reaction, which is indicative of ascomycetes (Kurtzman and Fell, 1998).

3.2.4.3 Molecular identification of yeasts

3.2.4.3.1. DNA extraction

Two millilitres overnight YM broth cultures of the yeast isolates were prepared and centrifuged to obtain a pellet of the cells. This was followed by genomic DNA extraction according to the modified method of Hoffman and Winston (1987). Cultures (2 mL) were centrifuged for one minute at 14,000 x g and the supernatant was removed by aspiration. Cells were suspended in 500 µL DNA lysis buffer (100 Mm Tris HCL at pH 8.0; 50 Mm EDTA; 1% SDS). Glass beads (200 µL) were added to the suspension, vortexed for 4 min and cooled immediately on ice. The suspension was centrifuged at 14,000 x g and the liquid phase was transferred to a sterile micro-centrifuge tube. A total of 275 µL ammonium acetate (pH 7.0) was added, vortexed for 5 minutes and incubated at 65 °C for 5 minutes followed by immediate cooling on ice. To this suspension 500 µL of chloroform was added and mixed. The mixture was centrifuged for 2 minutes at 14,000 x g at 4°C. The top layer was transferred to a sterile micro-centrifuge tube and 750 µL of isopropanol added. This was incubated for 5 min at room temperature. DNA was purified using the Nucleospin Tissue kit (Macherey-Nagel, Germany). The protocol of this kit was followed from step 5 onwards. Wash steps, drying of the membrane and elution of the DNA were followed as indicated in the Nucleospin protocol. Eluted DNA samples were stored at 4°C until further analysis.

A Nanodrop™ 1000 spectrophotometer (Thermo Scientific, US; NanoDrop, 2007) was used to quantify the DNA yield. The integrity of the extracted DNA was verified by gel electrophoresis (Jordaan and Bezuidenhout, 2013). DNA samples were stored at 4°C for short periods.

3.2.4.3.2. Amplification and sequence validation

The 26S rRNA gene fragments were amplified using the Polymerase Chain Reaction (PCR) procedure as described in Monapathi et al. (2017). Forward primers, NL1 (5-GCATATCAATAAGCGGAGGAAAAG-3) and reverse primers, NL4 (5-GGTCCGTGTTTCAAGACGG-3) (O'Donnell, 1993) were used. Electrophoresis was used to determine quality and PCR success. The 26S rRNA PCR products were purified as described by Li et al. (2010). Purified PCR products were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, UK) as prescribed by the manufacturer.

Clean-up of the sequencing reaction was achieved using the NaOAc/EDTA/Ethanol method (Applied Biosystems, UK) and sequenced using an ABI 3130 Genetic Analyser (Applied Biosystems, Hitachi). Procedures as described by Jordaan and Bezuidenhout (2013) were used. Chromatograms were viewed in Geospiza Finch TV (version 1.4) software, and BLAST (Altschul et al., 1997) searches (<http://www.ncbi.nlm.nih.gov/BLAST>) were used to determine the identity of the amplified sequences. Sequence reads obtained in the present study were submitted to GenBank. Some of the accession numbers provided are listed here (KY985792- KY985818), (KY778699- KY778715), (MF042170- MF042205), (MF277005- MF277027) and (MF462356- MF462371).

3.2.4.4. Antifungal susceptibility tests

Susceptibility tests were performed by the disc diffusion method (Bauer et al., 1966). Pure yeasts colonies were inoculated into 9ml of YM broth and incubated at 37°C for 24 hrs. Spread plates of yeast cultures were prepared on YM agar plates (Monapathi et al., 2017). Discs (Mast Diagnostics, UK) of known concentrations of antifungal agents were placed on the plates and incubated at 37°C. The following commonly used antifungal drugs (and concentrations) were used: fluconazole (FCN; 25 µg), econazole (ECN: 1 µg), ketoconazole (KCA: 15 µg), miconazole (MCL: 1 µg), metronidazole (MZ: 5 µg), flucytosine (FY: 1 µg), nystatin (NY: 100 µg). Diameters of zones of inhibition (in mm) were measured after 24hrs. When no inhibition zones were obtained the results were interpreted as completely resistant yeasts to the tested concentration. The diameters were compared to the values in CLSI standard M44-A2 (2009) for *Candida albicans*. Zone breakpoints and interpretative categories for antifungal agents were classified as resistant, intermediate resistant or susceptible.

3.2.5. Data and statistical analyses

Microsoft Excel (2011) was used to standardise raw data. Descriptive and one-way ANOVA followed by Tukey's HSD (Honestly Significant Difference) Test for unequal sample sizes were used for post hoc comparisons of physico-chemical and microbiological parameters taking into account seasonality of the sampling. Significant differences were denoted by different letters between variables. Redundancy analysis (RDA) was used to test for association between physico-chemical parameter measurements and yeast levels. Data output was provided as correlation biplots. The correlation significance level was set at $p < 0.05$.

3.3. Results and discussion

3.3.1. Physico-chemical parameters

The mean value of the physico-chemical parameters were compared to water quality standards defined in TWQR for agricultural uses (irrigation and livestock farming) (DWAF1996a; 1996b).

3.3.1.1. Temperature

Water temperatures in all the river systems were between 14 and 28°C (Figure 2a). For each river system, wet-warm seasons characterised by the higher temperatures (23-28°C) compared to the dry-cold season (14-18°C). No significant seasonal differences were seen between the river systems except for Crocodile West River where the temperature was consistently different from the other river systems in both seasons. In study conducted by Gondwe and Masamba (2016) along a river transect through the Okavango Delta, Botswana, temperatures recorded for wet and dry seasons were 25.96 ± 0.25 and 19.53 ± 0.42 °C, respectively. Significant temperature differences observed were the result of decreasing vegetation cover during the wet season. These findings were confirmed by Dallas and Day (2004). The authors state that the removal of riparian vegetation that provides shading to the river exposes the water to the sun thus increasing water temperatures. Dallas and Day (2004), further ascribed increased temperatures to the return of irrigational practices and agricultural drainage. Traoré et al., 2016 recorded maximum (25 °C) and minimum temperatures (18–19 °C) in rivers in the Vhembe District, South Africa. These river systems in the present and in the study by Traoré et al., 2016) were both exposed to farming practices that included irrigation. Temperatures measured in

aforementioned and the present study were within the expected acceptable ranges for relevance to the sampling period (Traoré et al., 2016).

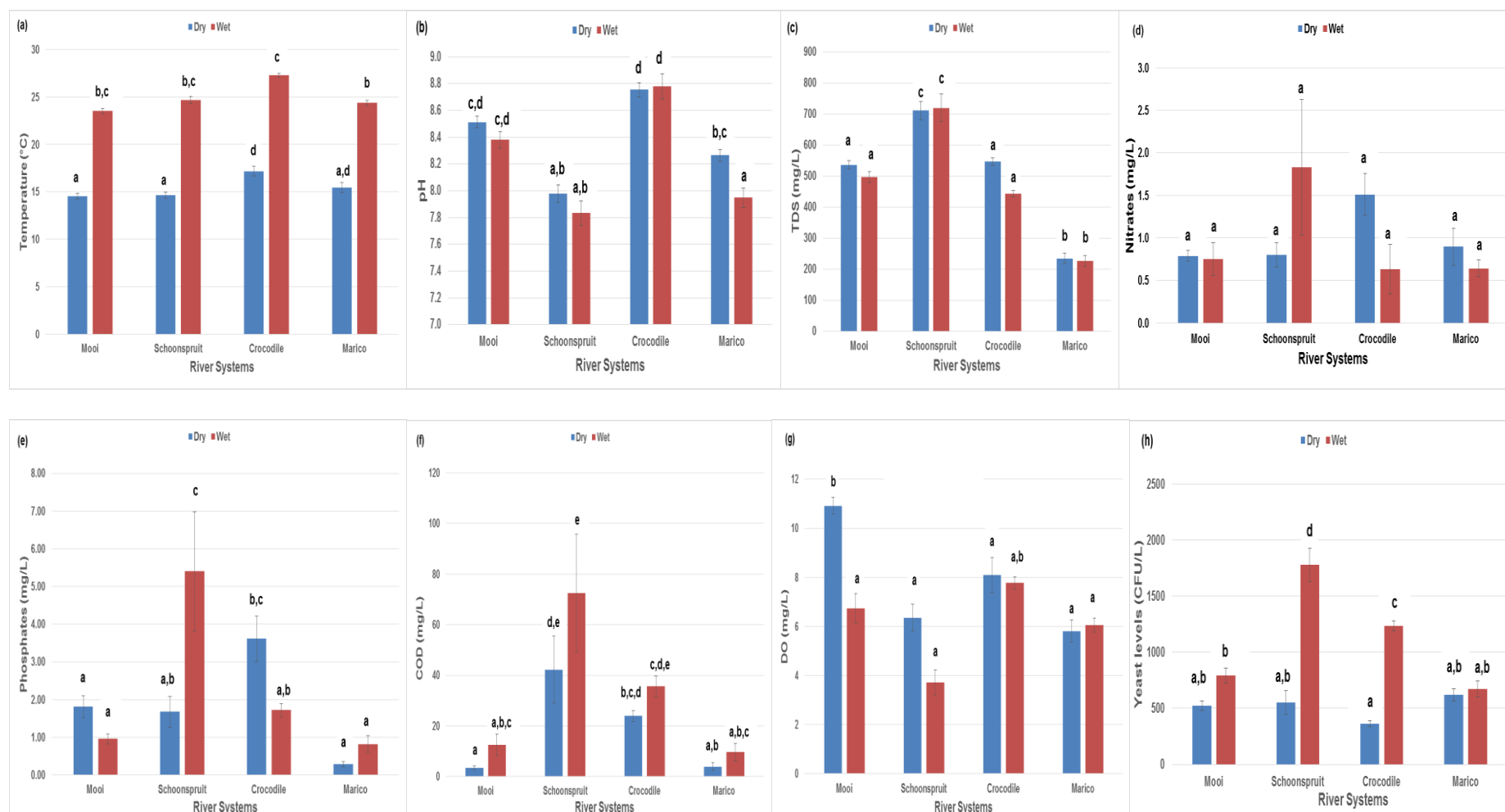


Figure 2. Mean values for physico-chemical and microbiological parameters in selected surface water resources in the NWP. (a) Temperature (°C), (b); pH, (c) Total Dissolved Solids (TDS) (mg/L), (d) Nitrates (mg/L), (e) Phosphates (mg/L), (f) Chemical Oxygen Demand (COD) (mg/L), (g) Nitrates (mg/L) and (h) Yeast levels at 37°C (CFU/L).

3.3.1.2. pH

The pH values for wet-warm and dry-cold season varied between 7 and 8 (Figure 2b). No significant seasonal differences in pH were observed for each river system except for Marico River. However, significant differences were observed between the river systems. The pH values were within the acceptable TWQR for its for irrigational use (6.5–8.4). Aquatic pH status in the present study could be associated with high concentration of CO₂ in the water column produced from the decomposition of organic matter. CO₂ dissolves in water to form the carbonic acid (H₂CO₃), which quickly dissociates into bicarbonate (HCO₃⁻) and proton (H⁺) ions, decreasing the pH (Schneider and Campion-Alsumald, 1999). Organic matter from anthropogenic pollutants discharged and ran off directly into the water body may cause the decrease in pH. On the other hand, photosynthesis decreases the concentration of CO₂ and HCO₃ in the water column (Schneider and Le Campion-Alsumald, 1999). Wet-warm seasons are associated with high photosynthesis rates thus high pH would be expected. The pH values recorded in this study are slightly alkaline. These results conform to previous studies by (Etim and Adie, 2012, Ayandiran et al., 2018). Findings from these authors established that the pH of surface water ranged between 6.5 and 8.5 and were within the standard regulatory limits (WHO, 2011).

3.3.1.3. Total dissolved solids (TDS)

Mean TDS values varied between wet seasons (226- 720mg/L) and dry seasons (235- 711mg/L) (Figure 2c). Although the maximum TDS was measured in the wet season, observed seasonal differences per river system were not significant. However, significant differences were observed between the river systems. These values were within TWQR for livestock farming (<1000mg/L) but above that

recommended for irrigational use (>40 mg/L). This was the case for all river systems. According to Chandra et al., (2011), TDS values higher than 500mg/L are not suitable for irrigation. However, DWAF (1996a) stipulates that TWQR ranges for irrigation (>540) can still be used in agriculture for selected crops provided sound irrigation management is adhered to. An efficient irrigation management strategy incorporates establishing the right times to irrigate, the amount of water to apply at each irrigational episode and proper operation and maintenance of the irrigation system (Holzapfel et al., 2009).

TDS comprises organic matter and inorganic salts, and other dissolved materials in water. These are naturally present in water or are discharges from mining, industrial and WWTPs or agricultural runoff (Weber-Scannell and Duffy, 2007, Muigai et al., 2010 Chandra et al., 2011,). TDS is a vital water quality indicator as the amount of solids (organic and inorganic contaminants) occurring in a water body can be used to estimate the pollution load (Ayandiran et al., 2018). High TDS have been measured in NWP due to natural causes and human impacts (NWP-SoER, 2014). Studies by Van Wyk et al., (2012) and Monapathi et al., (2017) recorded TDS as high as 950 mg/L in NWP surface water. The presence of dolomitic springs in the NWP could be responsible for high TDS in these river systems. The springs are characterised by abundant $[\text{CaMg}(\text{CO}_3)_2]$. The dolomitic eyes are also water sources for the river systems in the present study (Van Der Walt, 2002). High TDS values remain a public health threat. These affect the aquatic animals and plants by reducing fertilisation and productivity rates of fish, growth in algae and death (Leblond and Duffy, 2001, Dallas and Day, 2004).

3.3.1.4. Nutrients

During the wet-warm seasons the nitrate levels ranged between (0.6- 1.8 mg/L) and (0.7-1.51 mg/L) in the dry-cold seasons. No significant differences were observed seasonally per river system and between the river systems (Figure 2d). Phosphates concentrations for wet-warm seasons and dry-cold seasons recorded between (0.8- 5.4 mg/L) and (0.3- 1.8), respectively. No seasonal statistically significant differences were seen per river system except Schoonspruit River (Figure 2e). High nitrates and phosphates concentrations in water results from industrial discharges, sewage waste and fertilisers runoff from agricultural land (Abdul-Razak et al., 2009, Nyamangara et al., 2013). Dry land farming practices in the NWP might add significantly large amounts of phosphorus to the river in the form of fertilisers and animal manure (Jordaan and Bezuidenhout, 2016). Seasonal changes had no clear effect on the nutrients concentration in all the river systems.

The river systems in the present study are used for irrigation purposes in agriculture. However, the mean nitrates levels were above the permissible TWQR levels (<0.5 mg/L) for irrigational use. These elevated levels of nitrates results in recurrent growth of nuisance plants and blue-green algal blooms in irrigation structures (DWAF, 1996a). According to (DWAF, 2013b), the set standards for nitrates (<1.5 mg/L) and phosphates (<1.0 mg/L) were established as the special limits for domestic and industrial wastewater effluents that is allowed to decant into environmental waters. Nitrate mean values within wastewater quality standards were observed in all the river systems except Schoonspruit River (1.83 mg/L). Phosphate levels exceeded the quality standards in most sampling sites. According to US EPA, (1986), to control algal growth, the limit for phosphates is 0.1mg/L in flowing waters. Phosphate

concentration through the rivers in the present study exceeded the recommended limit. The maximum nitrates and phosphates value recorded in the wet-warm season could be due to runoff from urban and agricultural practices in the province.

Excessive nutrient loading in water resources is the major cause of eutrophication. This is a worldwide threat to water quality resulting in the loss of dominant species and functional groups, oxygen reduction, taste/odour generation in aquatic animals and plants and blooms of toxic algae (Liu and Qiu, 2007, Griffin, 2017). Cyanobacterial blooms are a serious public risk to fishing and state of surface water for irrigation, drinking and recreational use (Paerl, 2018). The algal blooms limit light penetration into the water and this affects the growth of aquatic organisms that require light for survival (Lehtiniemi et al., 2005). These organisms will consequently perish. As a result of eutrophication, photosynthesis reduces dissolved inorganic carbon and this raises pH to extreme levels during the day. Aquatic organisms that depend on oxygen for survival will be affected and subsequently die (Turner and Chislock, 2010). Eutrophication pollution effects involve the regulation of organic matter (C) and inorganic nutrients (primarily N and P) inputs into receiving waters (Pinckney et al., 2001). Under anoxic conditions, heterotrophic bacterial use organic carbon as food source. Moreover, these organisms reduce nitrate to more toxic (10-20 times) nitrite (Bouwman et al., 2005, Kirchman, 2012). Nitrite in water can cause substantial poisoning and diseases in aquatic organisms or humans (Razumov and Tyutyunova, 2001). Thus eutrophication will favour the survival of heterotrophic bacteria (Pinckney et al., 2001). Yeasts as heterotrophic organisms will also thrive under eutrophic water status. In studies conducted by Medeiros et al. (2008) and

Brandão et al. (2010) in eutrophic waters, high densities of yeasts were isolated due their ability to assimilate carbon sources from the environment.

3.3.1.5. Dissolved Oxygen (DO)

In the present study, DO concentration in water ranged from 3.7 to 7.8 mg/L during the wet-warm season and 6.4- 10.9 mg/L in dry-cold season (Figure 2f). No significant differences in DO in the dry seasons and in both seasons were seen in all the river systems. Significant difference seasonally was only observed in the Mooi River. DO is amount of dissolved oxygen in water. It is important to aquatic organisms for respiration. DO decrease in water would have adverse effects (Dallas and Day, 2004, Wilson, 2010). There exist a strong association between DO and eutrophication. Decomposition from algal biomass depletes DO and these may cause reduced oxygen availability in a body of water. Reduced oxygen in eutrophic water affects many aquatic organisms, and can result in fish death or forced migration (Hudnell, 2008). A study conducted by Gautam (2011) has linked DO to water pollution. At low DO (<4 mg/L), the stream water was considered polluted and could not be used for public supply, bathing, wildlife and fish culture. In this study, DO levels were above 4 mg/L in most of the river systems.

3.3.1.6. Chemical Oxygen Demand (COD)

Across the river systems, COD concentrations during the wet-warm season ranged between 9.6 and 72.5 mg/L and the dry-cold season was between 3.9 and 42.3 mg/L. These differences were however, not significant between the seasons. However, significant differences were observed between river systems (Figure 2g). COD reflects the total amount of oxygen equivalents that are consumed in oxidizing

organic compounds. Indirectly it measures the amount of organic compounds in water and this makes it a vital indicator of organic pollution (Ni et al. 2007, Alam, 2015). Wastewater limit value applicable to the irrigation of any land or property up to 2000 cubic metres for COD is <75 mg/L (DWAF, 2013b). Biochemical Oxygen Demand (BOD) not part of the present study is the amount of oxygen required by aerobic microorganisms to oxidise the organic matter. The source of the organic matter is WWTPs effluent or industrial waste (Mocuba, 2010). Under eutrophic status, oxygen consumed in the decomposition of organic matter cannot be utilised by aquatic organisms that require oxygen for survival and they could die. The biodiversity is affected as oxygen tolerant microorganisms will survive. COD and BOD are vital in determining water quality. High nutrient load will result in high COD and BOD, consequently high algal bloom in water (Manssour and Al-Mufti, 2010, Abdel-Raouf et al., 2012).

3.3.1.7. Yeast levels

Yeast levels were enumerated at room temperature (RT) and ranged from 928 to 3451 CFU/L. At 37°C levels were between 363 and 1778 CFU/L (Figure 2h). High yeast levels in the present study suggest pollution in the river systems. The number of yeasts increases in the presence of pollution or in the presence of algae, and it may reach a few thousand cells per liter or more (Hagler and Mendonça-Hagler, 1981). The highest yeast levels were measured during the wet warm season. This can be attributed to high temperatures and runoff from adjacent human activities polluting the river systems. No significant differences in yeast levels for all four river systems were observed during the dry season. Significant differences were observed between the wet warm seasons and cold dry seasons in Schoonspruit River and

Crocodile River. All of these observations were for enumeration at 37°C. Similar trends were observed when enumeration was done at RT. Virulence factors that characterise pathogenic yeasts include their ability to grow at elevated temperatures, particularly human body temperature of 37°C (Leach and Cowen, 2014). The ability of these environmental yeasts to grow at 37°C suggest that they could potentially be pathogenic.

3.3.2 Associations between physico-chemical water parameters and yeasts levels

Ecological factors such as temperature, pH, and freely available nutrients in the environment determine the metabolic activity, growth and survival of yeasts (Libkind et al., 2009, Yurkov et al. 2015). Mesophilic temperatures favour yeasts growth (Daek, 2006). Yeasts are heterotrophic organisms and thus require energy and carbon sources for growth. The energy is provided by the oxidation of organic molecules that also act as carbon sources for biosynthesis (Rodrigues et al., 2006, Messenguy et al., 2006). Redundancy analysis (RDA) ordination was employed to determine existing associations between yeast levels and physico-chemical parameters in the four river systems. The Mooi River biplot (Figure 3a) indicated that a positive association exists between yeast levels and three of the physico-chemical parameters (temperature (temp), pH and COD).

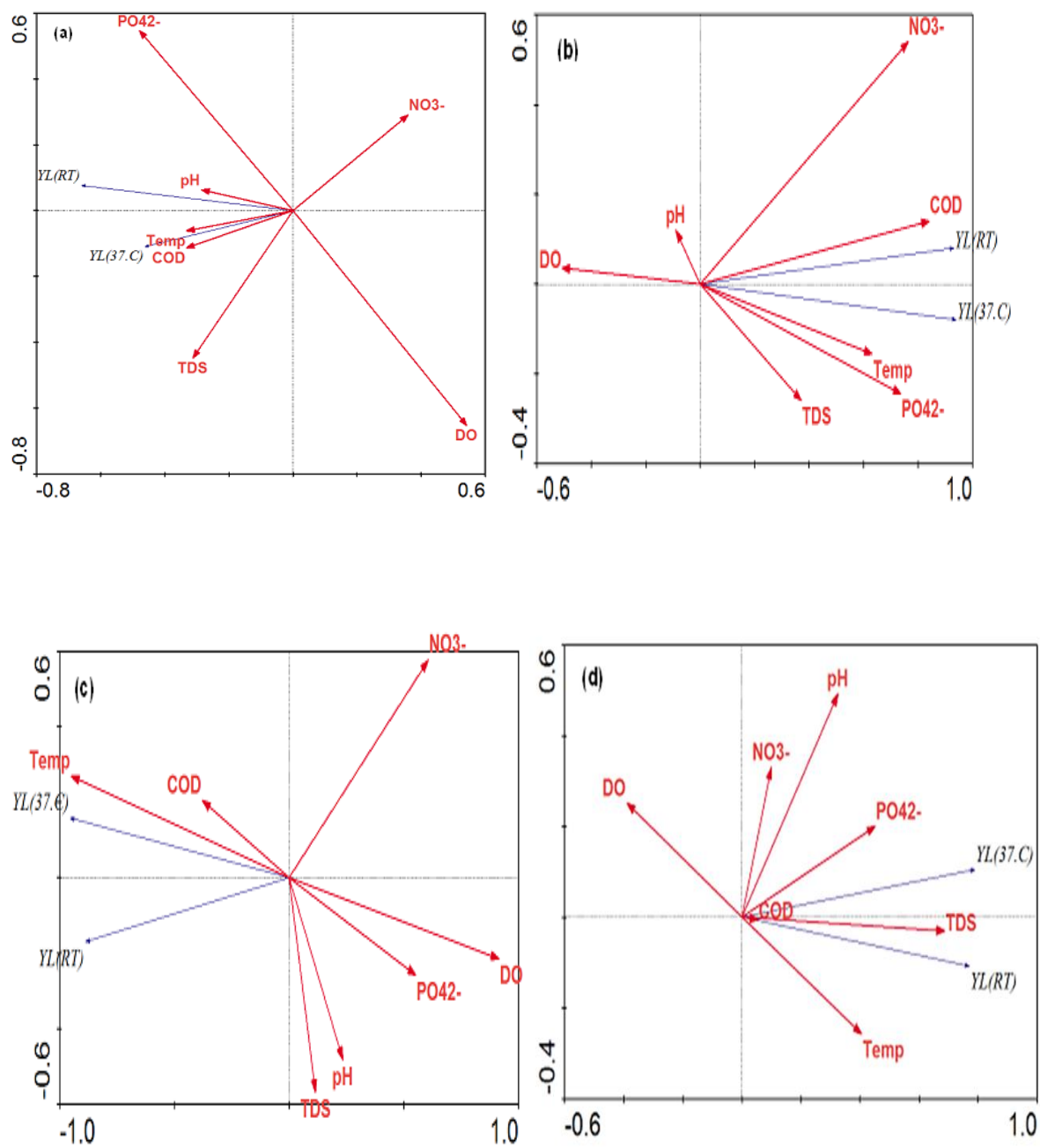


Figure 3. Redundancy analysis (RDA) ordination biplots illustrating correlation between environmental variables and yeast levels (YL) in various river systems. (a) Mooi River (b) Schoonspruit River (c) Crocodile River and (d) Marico River.

In the Marico River, a positive correlation of yeast levels with temperature, TDS, COD and phosphates was observed (Figure 3d). Although the COD levels affected the yeast levels this parameter had very little variation. Levels of yeasts in Schoonspruit River correlated with temperature, COD and phosphates (Figure 3b). For the Crocodile River a positive relationship is displayed between yeast levels and temperature and COD (Figure 3c). Dissolved oxygen and nitrates correlated negatively to yeast levels in all river systems.

Some studies have also established association between physico- chemical parameters and microbial status (Jan et al., 2014, Zhang et al., 2018). A study by Jan et al. (2014) in the Hokersar wetland, Kashmir Himalayas displayed a positive correlation of pH, temperature, nitrates and total phosphorus to yeasts levels in the wetland. From a study conducted by Zhang et al. (2018) in urban lakes, Xi'an, China, a positive correlation of COD, DO, total phosphorus, ammonium nitrogen and total nitrogen was observed. These authors also demonstrated that the mentioned parameters were associated with the abundance and structure of water fungal communities.

Although yeasts grow at higher temperature, in the present study, they could still grow in cold dry seasons. Temperature changes did not suppress yeast growth. According to Hagler and Mendonça-Hagler, 1979, alkaline pH does not favour growth of most yeasts. However, in the present study, fluctuations of pH values above neutral (pH 7.0) did not affect yeast growth. Yeasts as heterotrophic organisms have the ability to decompose organic matter in water (Rodrigues et al., 2006, Messenguy et al., 2006). The present study correlated high COD values with

high levels of yeasts. According to Dallas and Day, (2004), higher temperatures decreased the solubility of dissolved oxygen in water. A negative correlation between yeasts and DO confirmed low levels of DO at high temperatures.

It was expected that temperature will differ between the dry and the wet season. During the wet season the water temperature was significantly higher (Figure 2a) as these rivers are within a summer rainfall area. Except for phosphates in the Schoonspruit River (Figure 2d) and dissolved oxygen in the Mooi River (Figure 2f) there were no significant differences. Significantly higher yeast levels were observed in the Schoonspruit and Crocodile Rivers during the wet period compared to the dry season. These higher yeast levels are potentially due to the higher nutrients availability in the Schoonspruit River. Although not significant, the nutrient levels were lower during the wet season, compared to the dry season (Figure 2d,e).

3.3.3. Molecular identifications of yeast isolates

DBB staining classified yeasts as ascomycetous and basidiomycetous yeasts, with the former mostly isolated. The results correlates with most freshwater studies (Gadanhó and Sampaio, 2004, Medeiros et al., 2008, Pereira et al., 2009, Van Wyk et al. 2012, Brilhante et al., 2016). The number of yeast isolated varied, but this could be subjective. However, from the four rivers a total of 256 isolates were obtained and identified: (Mooi River = 99; Schoonspruit River = 84; Marico River = 43; Crocodile River = 30). Sequencing of 26S rRNA gene fragments revealed isolates representing 10 genera and 19 species. Table 2 is a summary of the various species, identification data and the rivers from which they were isolated. Sequence similarity searches identify homologous proteins or genes by detecting excess

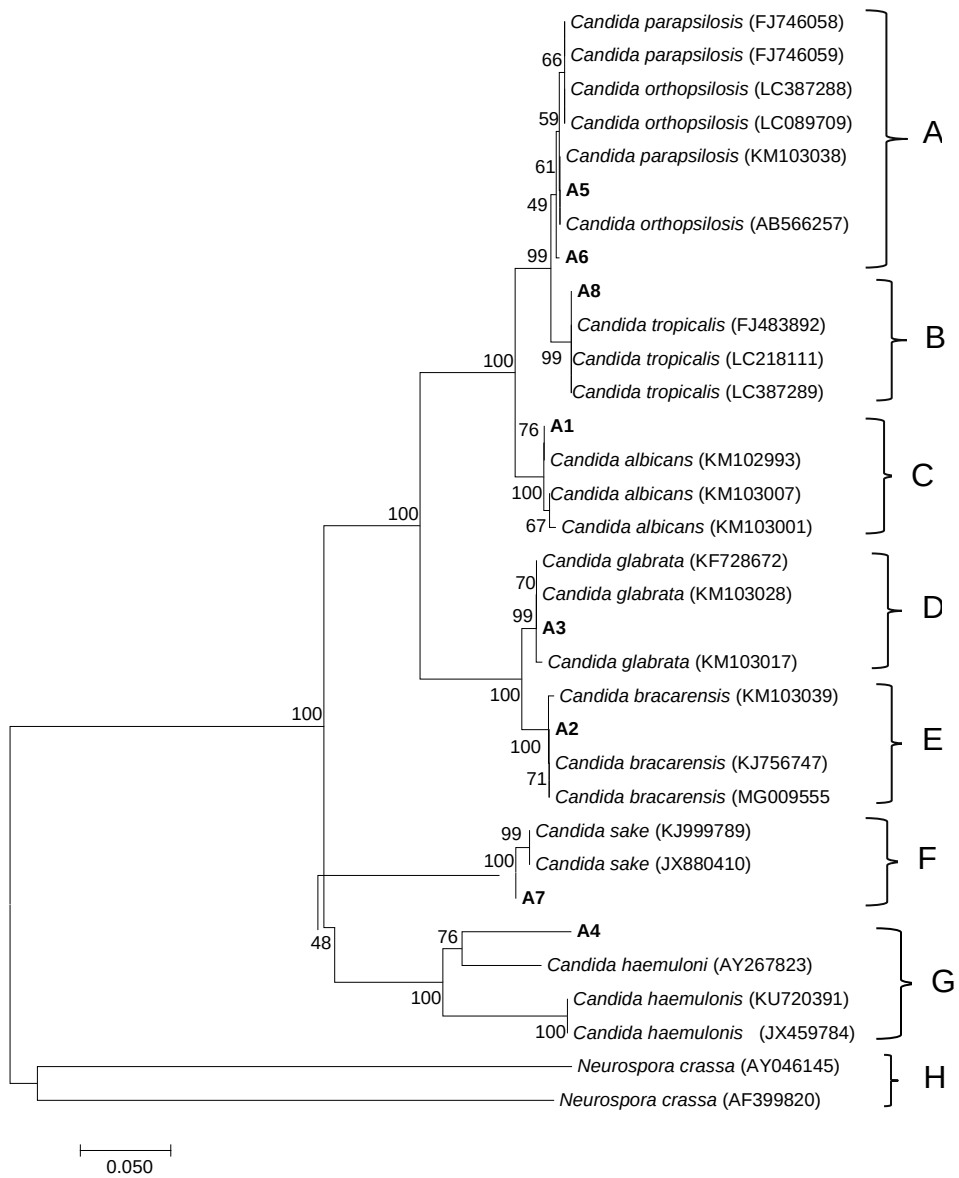
similarity (Pearson, 2013). A BLAST search conducted showed a high sequence similarity (>97%) between 26S rRNA gene sequences for most environmental isolates and the representative species from Genbank. Some yeast isolates had sequence similarity below 97% which is not acceptable for the identification of fungi (Raja et al., 2017). However, phylogenetic tree (Figures 3.4 a, b) showed high bootstrap value (>99%) which supported their identity (Paul et al., 2013). In descending order, the following numbers of genera were identified amongst the yeasts isolates: *Candida* spp. (36%), *Pichia* spp. (13%), *Cyberlinera* spp. (12%), *Meyerozyma* spp. (11%), *Clavispora* spp. (10%), *Saccharomyces* spp. (6%), *Kluyveromyces* spp. (5%), *Trichosporon* spp. (3%), *Yamadazyma* spp. (3%) and *Wickerhamomyces* spp. (1%).

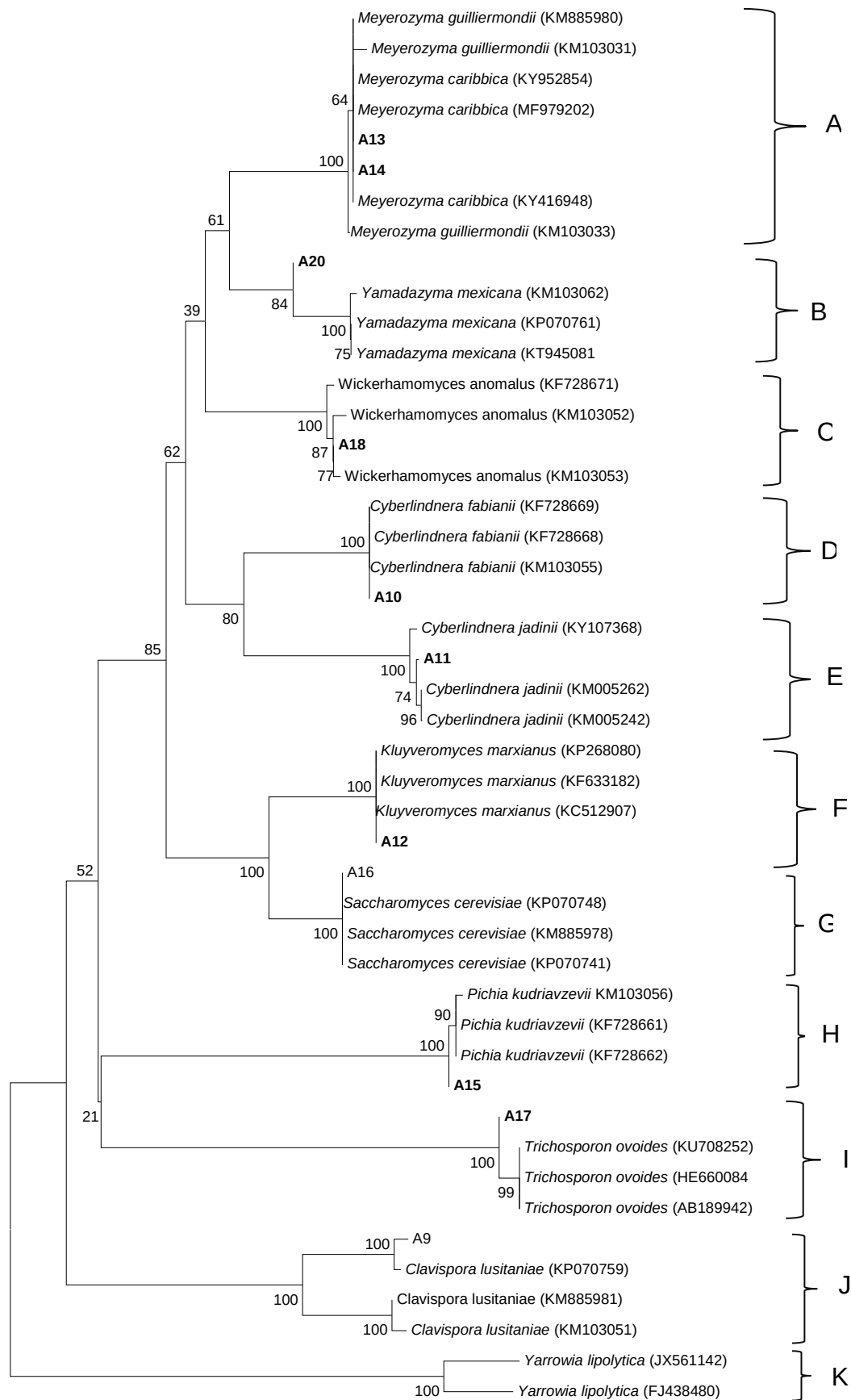
In the present study, the genus *Candida* was the dominant genus isolated (Table 2) and included the following species: *Candida albicans*, *C. bracarensis*, *C. glabrata*, *C. haemulonii*, *C. orthopsilosis*, *C. parapsilosis*, *C. sake* and *C. tropicalis*. This finding is similar to the findings of Medeiros et al. (2008) and Brilhante et al. (2016) in which they isolated and identified yeasts from freshwater environments. In their studies *Candida* sp. was also the dominant and included some of following: *Candida catenulata*, *C. glabrata*, *C. guilliermondii*, *C. melibiosica*, *C. krusei*, *C. parapsilosis*, *C. rugosa* and *C. tropicalis*. Non-*Candida* species isolated in the present study included: *Clavispora lusitaniae*, *Cyberlinera fabianii*, *C. jadinii*, *Kluyveromyces marxianus*, *Meyerozyma caribbica*, *M. guilliermondii*, *Pichia kudriavzevii*, *S. cerevisiae*, *Trichosporon ovoides*, *W. anomalus*, *Yamadazyma mexicana*. Most of these species have also been isolated from surface water environments (Medeiros et al., 2008, Pereira et al., 2009, Van Wyk et al. 2012, Brilhante et al., 2016, Monapathi et al., 2017). Table 2 shows some of the yeasts isolated from non-clinical

environments. Included in these are water, soil, plants and animals (CBS database, 2007).

Figures 3.4 a,b show a Neighbour-Joining Tree demonstrating a phylogenetic analysis between yeast species. Medium to high bootstrap confidence of 66 to 100% supported the relationship between the environmental isolates (bolded) and the representative 26S rRNA gene sequences obtained from GenBank. The bootstrap support is based on 1,000 replicates. A phylogenetic tree constructed from sequences of *Candida* species (Figure 4a) showed the formation of distinct clusters for each species. These species are clustered differently as they are phylogenetically distant. Each cluster indicates high sequence similarity between the groups (Ragonnet-Cronin et al., 2013). In the present study, same species from environmental isolates and representatives from GenBank formed one cluster except cluster A that comprised of two species: *Candida orthopsilosis* (formally known as *C. parapsilosis* group II) and *Candida parapsilosis*. *C. orthopsilosis* acquired a new name because PCR products from the groups differed in sizes, cluster analysis of sequence polymorphisms separated the isolates and DNA sequence similarities <90% in the ITS1 sequence (Tavanti et al., 2005). Clustering of similar species was also observed in non-*Candida* species (Figure 4b). Clusters H and K are out groups for *Candida* and non-*Candida* groups, respectively. Pathogenic species did not form a monophyletic group and were scattered all over the trees. This indicated that pathogenicity amongst these species evolved independently on multiple occasions (Diezmann et al., 2004).

(a)





(b)

Figure 4 A Neighbour-Joining Tree showing the phylogenetic relationship between environmental *Candida* species (a) and Non *Candida* species (b) (**bold**) and representative species from GenBank. A bootstrap test (1,000 replicates) was conducted and the cluster percentage of trees supporting the cluster is provided.

Yeasts are responsive to organic contamination (Brandão et al. 2010). Human activities adjacent to the water resource may influence the type of yeasts present in water. From earlier studies, yeasts were used as organic pollution indicators in aquatic environments (Nagahama, 2006). In the present study, some of the isolated species have been linked to polluted water resources. Woollett and Hendrick (1970) isolated *Candida* spp. from heavy industrial and domestic waste polluted water. A study by Rosa et al. (1995) associated *Candida* and *Saccharomyces* species with eutrophic waters. Various specific yeasts species such as *Candida albicans*, *C. famata*; *Cryptococcus laurentii*, *Pichia guilliermondii*, *W. anomalus*, *Rhodotorula glutinis*, *R. mucilaginosa* and *Trichosporon beigeli* have also been implicated in industrial and wastewater pollution (Hagler, 2006, Nagahama, 2006, Vogel et al., 2007, Medeiros et al., 2008). The occurrence of *S. cerevisiae* in freshwater environments has been linked water polluted from water industrial activities (Hagler, 2006, Brandão et al., 2010).

As indicated in Table 2, most of the yeast species (74%) isolated in this study are clinically relevant. The same species were also isolated from yeast infections in humans (CBS database, 2007). These are predominantly, *Candida* species, the most important disease causing agent in human (Puebla, 2012). Highly lethal

invasive infections caused by *Candida* species include disseminated candidiasis, endocarditis, meningitis, and hepatosplenic infections. These infections are found in the lungs, brain and bloodstream. These are major causes of morbidity and mortality in the healthcare environment (Pfaller et al., 2011, Scorzoni et al., 2017). *Candida* species are also responsible for non-invasive mucosal infections in the oropharynx, oesophagus and the vagina (Vandeputte et al., 2012, Pappas et al., 2016). According to ARTEMIS Global Antifungal Surveillance Program, main prevalent agents of candidiasis in descending order comprise of *Candida albicans* (63-70%), *C. glabrata* (44%), *C. tropicalis* (6%) and *C. parapsilosis* (5%) (Pfaller et al., 2007). These findings concurred with a study by Miceli et al. (2008) and Pappas et al., (2016). *C. albicans* is the most common yeast pathogen in immunocompromised people and patients receiving immunosuppressive therapy (Pongrácz et al., 2015). It is mostly found in intensive care unit patients and cause infections that can lead to severe sepsis and septic shock (Kollef et al., 2012).

The notion by Pfaller et al. (2007) on *C. albicans* as the dominant species in patients with invasive candidiasis has changed. Epidemiology have shown non-albicans species *C. glabrata* and *C. parapsilosis* independently constituted about half of the isolates on the studies conducted (Arendrup, 2014, Guinea, 2014, Puig-Asensio et al., 2014). From a study by Savastano et al. (2016) in hospital environment, 91% of the isolates were identified non-albicans species and included *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. lusitaniae* and *C. famata*. Nosocomial infections caused by yeasts are a public health threat and are typically found amongst patients admitted in hospitals. Some of the above-mentioned species were also isolated in the present study.

Pathogenic non-*Candida* spp. isolated in the present study are *Clavispora lusitaniae*, *Cyberlinera fabianii*, *Meyerozyma caribbica*, *M. guilliermondii*, *S. cerevisiae*, *T. ovoides*, *W. anomalus*. A study by Yamamoto et al., (2018) has reported on endophthalmitis, a bloodstream infection caused by *C. lusitaniae*. A close relationship between *Meyerozyma caribbica* and an opportunistic human pathogen *M. guilliermondii* suggests it may be an occasional clinical pathogen (Vaughan-Martini et al., 2005). *M. guilliermondii* has been reported mostly as a cause of candidemia in patients with cancer (Savini et al., 2011). *S. cerevisiae* classically regarded safe non-pathogenic yeast has been implicated in infections from vaginitis in healthy patients. Furthermore, it has caused cutaneous infections and systemic bloodstream infections in immunocompromised patients (de Llanos et al., 2011). *Trichosporon ovoides* causes superficial trichosporonosis, a skin and hair infection in immunocompromised people (Silvestre et al., 2010). *W. anomalus*, hardly considered a causal agent in human infections has been catheter-related, neonatal and ocular infections (Murphy et al., 1986, Klein et al., 1988, Hanada et al., 2012).

3.3.4. Antifungal resistance pattern

Table 3 depicts the number of yeasts that show complete resistance to antifungal agents. All yeast isolates (100%) were completely resistant to metronidazole and flucytosine. These were followed by fluconazole (79%), econazole, miconazole and ketoconazole (49%) and nystatin (15%). Table 2 illustrates that pathogenic yeasts *Candida albicans*, *C. glabrata*, *C. haemulonii*, *C. orthopsilosis*, *C. tropicalis*, *Clavispora lusitaniae* and *Trichosporon ovoides* showed 100% resistance to azoles. This is a worrying factor as azoles are the most commonly used antifungal drugs.

They are significantly favoured due to their broad spectrum of activity, safety profiles and costs (Li et al., 2014). *Candida* spp. in the present study were all susceptible to nystatin except *Candida parapsilosis*. This result was contradictory to a clinical study by Miranda-Cadena et al., (2018) where *Candida* spp. were resistant to nystatin.

Table 2. Distribution of yeasts species in the rivers systems, identification data, source and their % resistance to antifungal agents (Y= Yes; N= No; C= Clinical source; NC= Non Clinical source).

Yeast species	Code name (no of isolates)	Minimum % similarity	Pathogenic	Environmental source	River systems				Antifungal agents (%resistance)						
					Mooi	Schoonspruit	Crocodile	Marico	FCN	ECN	KCA	MCL	MZ	FY	NY
					River	River	River	River							
<i>Candida albicans</i>	A1 (34)	96	Y	C/NC	21	3	4	6	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>Candida bracarensis</i>	A2 (2)	100	Y	C/NC	0	0	2	0	100.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>Candida glabrata</i>	A3(37)	91	Y	C/NC	14	14	7	5	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>Candida haemulonii</i>	A4 (3)	88	Y	C/NC	0	0	0	3	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>Candida orthopsilosis</i>	A5 (3)	99	Y	C/NC	0	0	0	2	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>Candida parapsilosis</i>	A6 (3)	99	Y	C/NC	1	2	0	0	0.0	0.0	0.0	0.0	100.0	100.0	100.0
<i>Candida sake</i>	A7 (3)	99	N	NC	1	2	0	0	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>Candida tropicalis</i>	A8 (4)	98	Y	C/NC	4	0	0	0	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>Clavispora</i>	A9 (26)	97	Y	C/NC	14	10	0	2	100.0	100.0	100.0	100.0	100.0	100.0	0.0

lusitaniae

<i>Cyberlinera</i>	A10 (25)	100	Y	C/NC	12	12	0	1	0.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>fabianii</i>															
<i>Cyberlindnera</i>	A11 (7)	98	N	NC	3	2	0	2	0.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>jadinii</i>															
<i>Kluyveromyces</i>	A12(13)	99	N	NC	5	7	0	1	0.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>marxianus</i>															
<i>Meyerozyma</i>	A13 (7)	99	Y	C/NC	2	2	0	3	100.0	0.0	0.0	0.0	100.0	100.0	100.0
<i>caribbica</i>															
<i>Meyerozyma</i>	A14 (21)	96	Y	C/NC	4	7	4	6	100.0	0.0	0.0	0.0	100.0	100.0	100.0
<i>guilliermondii</i>															
<i>Pichia kudriavzevii</i>	A15 (32)	98	N	NC	10	14	4	4	100.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>Saccharomyces</i>	A16(15)	99	Y	C/NC	3	7	3	2	100.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>cerevisiae</i>															
<i>Trichosporon</i>	A17(8)	99	Y	C/NC	3	0	2	3	100.0	100.0	100.0	100.0	100.0	100.0	0.0
<i>ovoides</i>															
<i>Wickerhamomyces</i>	A18 (3)	99	Y	C/NC	0	0	0	1	0.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>anomalus</i>															
<i>Yamadazyma</i>	A20(10)	99	N	NC	2	2	4	2	0.0	0.0	0.0	0.0	100.0	100.0	0.0
<i>mexicana</i>															

Table 3. Number of isolated yeasts from surface water resources in the NWP that showed antifungal resistance to various commonly used antifungals (FCN= Fluconazole; ECN= Etonazole; KCA= Ketoconazole MCL= Miconazole MZ= Metronidazole; FY= Flucytosine; NY= Nystatin)

Sampling site	Antifungal agents						
	FCN	ECN	KCA	MCL	MZ	FY	NY
Mooi River	78/99	58/99	58/99	58/99	99/99	99/99	6/99
Schoonspruit River	59/84	35/84	35/84	35/84	84/84	84/84	11/84
Marico River	38/43	21/43	21/43	21/43	43/43	43/43	9/43
Crocodile River	26/30	13/30	13/30	13/30	30/30	30/30	8/30
Total	201/256	127/256	127/256	127/256	256/256	256/256	39/256
%	79	49	49	49	100	100	15

Several studies have also reported on environmental yeast resistance to antifungal drugs (Brandão et al., 2010, Medeiros et al., 2008, Monopathi et al., 2017). Medeiros et al., (2008) conducted a study in tropical freshwater environments in South-eastern Brazil. The water resource was polluted by rapid urbanisation and lack of basic sanitation. Antifungal drug susceptibility tests showed fifty percent of the yeast isolates resistant to itraconazole. Pathogenic yeasts, *Candida tropicalis* and *Candida krusei* were resistant to all used antifungal agents: ketoconazole, fluconazole, itraconazole, terbinafine and amphotericin B. Brandão et al. (2010) conducted a study in three lakes in the paleokarstic region of the Lagoa Santa plateau in the state of Minas Gerais in South-eastern Brazil. The water quality in these lakes was affected by poor sanitation, eutrophication, growing urbanisation and increasing siltation. Isolated pathogenic yeasts were subjected to antifungal susceptibility tests. High yeast resistance to amphotericin B (21.7%), followed by itraconazole (20%) and fluconazole (2.8%) was observed.

Monopathi et al. (2017) conducted antifungal resistance analysis from two rivers in the NW, viz. the Mooi River and Harts River. Isolates were obtained during two sampling seasons in 2014 and 2015. These yeast isolates from both rivers, were all resistant to miconazole and flucytosine. A large proportion of the isolates were resistant to fluconazole (88.5 and 92.5%, from Mooi and Harts Rivers, respectively). In Mooi River, 62.8% of the isolates were resistant to both econazole and miconazole and 64.1% were resistant to ketoconazole. In the Harts River, 12.5% of the isolates were resistant to the three azole antifungal drugs (Monopathi et al., 2017). These results showed some similarities to the present study in which azole resistance was also common amongst the isolates from four rivers in the NWP.

Resistance patterns that were observed are a cause for concern. Aquatic environments hosting these pathogenic antifungal resistant yeasts are a public health threat. They pose a serious risk to people who use the water for recreation or other uses.

Antifungal agents used in the study are commonly used to treat invasive yeast infections in immunocompromised people such as HIV patients. With high HIV infection globally (36.7 million) and South Africa (7.06 million) (Statistics S.A, 2017, UNAIDS 2017), resistance by pathogenic yeasts to these antifungal agents place community health at risk. The NWP was previously reported to have an HIV infection rate of 13% (Shisana et al., 2014). The community uses the water for irrigation, mining, recreational and religious purposes (DWAf 2004, Van Aardt and Erdman, 2004, NWP-SoER, 2014). Direct contact with water could cause yeast infections in especially immunocompromised people. These people are likely to be affected by yeasts infections ranging from superficial (thrush, athlete's foot and ring-worm) to life threatening infections that invade the blood stream, disseminate to internal organs and cause meningitis and candidiasis (Gould, 2012, Whaley et al., 2016).

The mode of action and resistance mechanisms by azoles used in medicine and agriculture are similar (Ribas et al., 2016). However, resistance mechanisms against antifungals have largely been studied for clinical isolates. Resistance mechanisms of yeasts to antifungal agents has been determined at molecular level in pathogenic yeasts such as *Candida albicans*, *C. glabrata*, *C. parapsilosis* and *Cryptococcus neoformans* (Garcia-Effron et al., 2008, Sanglard et al., 2009, Kofla et al., 2011, Mario et al., 2012, Brilhante et al., 2016, Bosco-Borgeat et al., 2016). The

mechanisms identified include the presence of efflux pump transporter genes, alteration of the gene coding for the target enzyme (14 α -lanosterol demethylase) and use of differential pathways (C-5 sterol desaturase in the ergosterol biosynthesis pathway (Cowen et al., 2015, Khosravi Rad et al., 2016, Salari et al., 2016, Sanglard et al., 2016). Resistance mechanisms using efflux pump transporter genes were found to also exist in environmental *Candida albicans* (Monapathi et al., 2018). One or a combination of the abovementioned mechanisms could potentially cause antifungal resistance in the environmental yeast species isolated in the current study. If these would cause infections in humans then it could be challenging to treat.

3.3.5 Conclusion

The mean values of the physico-chemical parameters were within TWQR for livestock farming but out of range for irrigation. This is a concern because the river systems are largely used for irrigation and the observed levels of these parameters could have implications for food production and food security. The Schoonspruit River was the most polluted river system characterised by high yeast levels, TDS, COD, nitrates and phosphates. Results presented here demonstrate the attractiveness of using yeasts as a microbiological indicator of organic pollution of water ecosystems. Significant differences in physico-chemical parameters determined the influence of environmental factors on the water quality seasonally per river system and between the rivers. This aspect was not specifically focussed on in the present study but should be considered in future studies. The presence of pathogenic species and resistance to the antifungal drugs shows that yeasts could pose a health risk to the people especially immunocompromised people who use the river water directly for different activities. Further studies to increase the data sets in

which yeasts are used, in conjunction with other water quality parameters should be conducted. Resistance to antifungal drugs is a health threat as it renders treatment difficult. To minimise antifungal resistance, regulated use of antifungals in prophylactic, veterinary agricultural use should be properly enforced. Furthermore, proper treatment of agricultural wastewater should be done. Wastewater treatment plants must be properly managed as wastewater effluent may contain antifungals that were used by communities. More studies on antifungal resistance mechanisms should be conducted to find solution against resistance and to validate the development of newer antifungal drugs.

CHAPTER 4

Efflux pumps genes of clinical origin are related to those from fluconazole-resistant *Candida albicans* isolates from environmental water

4.1 Introduction

Yeast species have been isolated from North West Province (NWP) Rivers (Van Wyk et al., 2012, Monapathi et al., 2017). Some of these are known human pathogens/opportunistic pathogens (Yamaguchi, 2007). *Candida albicans* (*C. albicans*), the most notably known opportunistic pathogen (Ahearn, 1998) has been isolated from NWP surface water resources (Van Wyk et al., 2012, Monapathi et al., 2017). In healthy people, it is a commensal, harmless colonizer of mucosal surfaces (Achkar and Fries, 2010). However, in immunocompromised people such as cancer and transplant patients as well as those infected by the HIV, it causes superficial as well as life threatening systemic infections (Fridkin and Jarvis, 1996, Morschhäuser, 2002, Mayer et al., 2013). The presence of *C. albicans* in surface water environments has been linked to polluted waters (Cook and Schlitzer, 1981, Van Wyk et al., 2012, Monapathi et al., 2017). This is a concern as people in the NWP use surface water for different purposes that involve direct contact such as recreation as well as religious activities (NWP-SOER, 2014). In such direct contact with water, immunocompromised individuals are at risk of being infected with *C. albicans*. In the North West Province the HIV prevalence rate, has estimated to be at 13% (Shisana et al., 2014).

Antifungal agents are used to treat mycotic infections. These are categorized into different classes based on their mechanism of action. The various classes include the azoles, polyenes and pyrimidines. Azoles are mostly preferred class of antifungals in mycotic therapy (Mansfield et al., 2010). Fluconazole, an azole derivative is the antifungal of choice in South Africa (Truter and Graz, 2015) and is part of the prophylactic treatment in HIV positive patients to prevent increasing infections by *Candida* spp. and *Cryptococcus* spp. (Morschhäuser, 2002, Abrantes et al., 2014). The latter species (*Cryptococcus* spp.) cause cryptococcosis, one of the most common opportunistic infections and causes of death among HIVinfected patients (Srichatrapimuk and Sungkanuparph, 2016). Azoles enter the yeast cells by facilitated diffusion (Mansfield et al., 2010) and inhibits lanosterol 14 α -demythylase an critical enzyme in the ergosterol biosynthetic pathway (Akins, 2005). Ergosterol is major sterol in fungal membranes responsible for fungal cell growth and proliferation as well as fluidity and integrity of the cell membrane (Joseph-Horne and Hollomon 1997, White et al, 1998). The enzyme inhibition leads to depletion of ergosterol as well as the production of a toxic methylated sterol. Consequently, the membrane functions are impaired (Morschhäuser, 2016).

Fluconazole is also mostly used to treat *Candida* infections due to its favourable bioavailability and safety outline (Rex et al., 1995, Morschhäuser, 2002). However, fluconazole is not completely metabolized in the body and it is excreted in the urine and eventually lands at wastewater treatment plants (WWTPs) (Kim et al., 2007, Kahle et al., 2008, Kim et al., 2009). Most WWTPs in the NWP are poorly managed (DWAf, 2013a) and sewage may not be properly treated. Consequently antifungal

drugs and other antimicrobial chemicals may not be removed by the WWTP processes and could end up in surface water system. Antifungals can also be introduced into the water resources through run off from agricultural settings where azoles are used to protect grain crops from fungal diseases (Mateo et al., 2013, Singer et al., 2016).

Prophylactic and continuous use of fluconazole resulted in the development of yeast strains that are resistant to this and related drugs (Ruhnke et al., 1994, Monapathi et al., 2017). For clinical relevant *C. albicans*, several molecular mechanisms that explain resistance to fluconazole have been documented. These include the most frequent multidrug transporters encoded by the active efflux pump genes (*CDR1* and *CDR2*, *FLU1* and *MDR1*), alteration (either by mutation or by overexpression) of the target enzyme, 14 α -lanosterol demethylase. The latter enzyme is encoded by the gene *ERG11* (Sanglard et al., 2016). On the other hand, the inactivation of the sterol C5.6-desaturase is encoded for by *ERG3* gene. The product of the latter gene (*ERG3*) is also involved in the ergosterol biosynthesis pathway (Cowen et al., 2015, Sanglard et al., 2016). A series of isolates from individual patients have shown an amalgamation of several of these mechanisms that could result into a stepwise development of clinically relevant fluconazole resistance (Morschhäuser, 2002, White et al., 1998).

Efflux pumps mediated resistance is the dominant drug resistance mechanism seen in yeasts from human immunodeficiency virus infected patients (Perea et al., 2001).

The resistance mechanism uses transporter protein pumps to transport toxic substances as well as drugs across the fungal plasma membrane to the external environment. The accumulation of drugs in the cells is reduced (Cernica and Subik, 2006, Webber and Piddock, 2003). *Candida* drug resistant (*CDR1* and *CDR2*) are genes that encode ATP- binding cassette (ABC) transporters. Multidrug resistance (*MDR 1*) and fluconazole resistance (*FLU 1*) genes encode major facilitator (MF) drug pumps. MFs use a proton gradient across the membrane as the energy force (Morschhäuser, 2002).

Studies conducted in the North West Province have reported the presence of the microorganisms that are resistant to several clinically relevant antimicrobial agents (Mulamattathil et al., 2014, Molale and Bezuidenhout, 2016, Monapathi et al., 2017). The emphasis on efflux pumps as resistant tools has mainly been placed on clinical isolates as these pose a higher public health threat (Dada et al., 2013). Little attention has been brought to environmental isolates. Molale and Bezuidenhout (2016) has determined efflux pump as the resistance mechanism in *Enterococcus* species isolated from surface water systems. Mechanisms involved in antifungal resistance have not been determined in antifungal resistant yeast from the NWP. The aim of this study was to determine antifungal susceptibility of environmental *C. albicans* and to establish whether (i) efflux pump genes are present in these isolates; and (ii) these are genetically different or similar to clinical isolated strains

4.2 Materials and methods

4.2.1 Study design

Antifungal resistant *C. albicans* were isolated from the NWP Rivers during 2015 and 2016. This was a follow up study to that described by Monapathi et al. (2017). Water samples were collected from the Mooi River, Harts River, Schoonspruit River, Crocodile River as well as the Marico River (Figure 5). The impacts on the rivers are pollution from domestic, agricultural and mining activities (Bezuidenhout et al., 2013). However, the water is also used in industries, mining, agriculture, domestic and religious purposes (Coetzee et al., 2016, Molale and Bezuidenhout, 2016).

4.2.2 Samples collection and yeast isolation

Water samples were collected aseptically using the direct and dip sampling technique. Membrane filtration was performed to determine the levels of yeasts in water. For this, the membranes were incubated on YM agar supplemented with 100ppm chloramphenicol. Incubation was at 37°C for 24 hrs. Successive streak plating on YM was done to obtain pure colonies (Wickerham, 1951, Van Wyk et al., 2012).

Sampling River systems running through the North West Province

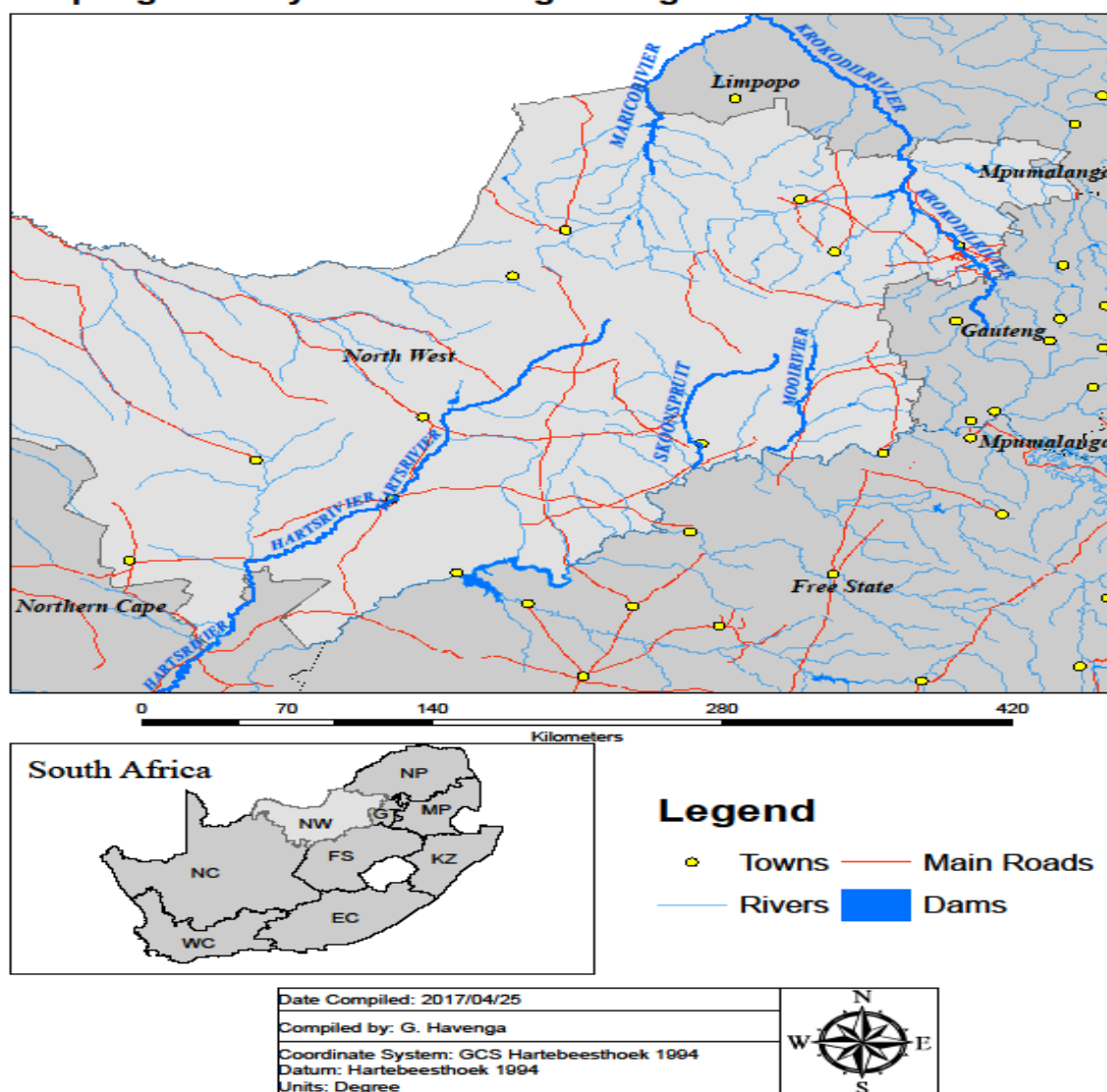


Figure 5. Map showing geographical location of selected rivers in the North West Province and neighbouring provinces.

4.2.3 Molecular identification and antifungal susceptibility tests

Two millilitres overnight YM broth cultures of the yeast isolates were prepared and centrifuged to obtain a pellet of the cells. Extraction of the genomic DNA was done according to the modified method of Hoffman and Winston (1987) (Monapathi et al.,

2017). DNA samples were stored at 4°C for short periods. DNA concentrations were determined using the Nanodrop spectrophotometer (Thermo Scientific, US) (NanoDrop, 2007). Gel electrophoresis was done to determine the quality and quantity of the extracted DNA. Molecular identification was performed as described in Monapathi et al. (2017). All the 26S rRNA sequences were deposited into GenBank. Some of the provided accession numbers were (KM102991-KM102997), (KM103005-KM103007) and (MF042197-MF042198). The sequences were subjected to phylogenetic analysis (Monapathi et al., 2017).

The Kirby Bauer Disk diffusion method was performed according to CLSI standard M44-A2 (CLSI, 2009) to determine antifungal susceptibility of the *C. albicans* isolates.

4.2.4 Detection of efflux mediated resistant genes

End-point PCR was used to determine the presence of resistant genes: *CDR1*, *CDR2*, *FLU1* and *MDR1* in fluconazole resistant *C. albicans* isolates. To amplify these resistant genes, the following primers were used: *CDR1* and *CDR2* fwd (5'-TATGTCAGATTCTAAGATGTC -3') and *CDR1* and *CDR2* rev (5'-TCGATACCTTCACCTCTG -3): *FLU1* fwd (5'- CAACGATATTGCTCCTGAAG-3') and *FLU1* rev (5'-TGGCTCTTCTCGATAATTCA-3'): *MDR1* fwd (5'-TTACCTGAACTTTTGGCAAAACA-3') and *MDR1* rev (5'-ACTTGTGATTCTGTCGTTACCG-3') (Mukherjee et al., 2003, Chau et al., 2004, Li et al., 2013). PCR was carried in a total volume of 25 µl. PCR reagents consisted of : (i) 12.5 µl double strength Amplitaq Gold® 360 Master Mix (Applied Biosystems, USA) (AmpliTaq Gold DNA Polymerase, 0.05 U/µl, GeneAmp PCR Gold Buffer, 30

mM Tris/HCl, pH 8.05, 100 mM KCl, dNTP, 400 µM each, MgCl₂, 5 mM), (ii) 2µl of (~ 50 ng) genomic DNA template, (iii) 3.0 µl primer mix (10 µM) and (iv) 7.5 µl nuclease free water, Thermo Scientific, Life Sciences, US). PCR conditions consisted of an initial denaturation (600 seconds, 95°C) followed by 35 cycles of denaturation (30 seconds, 95°C), annealing 30 seconds at 52°C (*CDR1*, *CDR2* and *MDR1*) or 54°C (*FLU1*) and extension (90 seconds at 72°C). This was followed by a final extension step (420 seconds, 72°C).

4.2.5 Determination PCR successes

Two microliters of amplified DNA was examined in a horizontal agarose gel (1% (w/v)) containing ethidium bromide (0.1 µg/mL) using 1X TAE buffer [(20 mM acetic acid (Merck, US); 40 mM Tris (Sigma Aldrich, US); 1 mM EDTA (Merck, US), pH 8.0)] as the electrophoresis buffer. Electrophoresis was conducted at 80 volts for 45 min and viewed using a ChemiDoc™ (BioRad, US) imager.

4.2.6 Phylogenetic analysis on resistance genes

The sequences of the amplification gene products were determined (Monapathi et al., 2017). These were submitted to GenBank and accession numbers for the genes are: *CDR1/CDR2* (KY979111-KY979117), *MDR1* (MF042166-MF042169), *FLU1* (MF115144-MF115149). A number of clinical representative *CDR1*, *CDR2*, *FLU1* and *MDR1* yeast gene sequences were downloaded from GenBank to compare their phylogenetic relationship with the gene sequences from the environmental yeast isolates. Multiple sequence alignment were done using Clustal W version 1.8 (Thompson et al., 1994). DAMBE was used to edit aligned sequences (Xia and Xie,

2001). Neighbour-Joining method in MEGA version 7.0 software (Kumar et al., 2016b) was used to construct a phylogenetic dendrogram.

4.3 Results

4.3.1 Molecular yeast species identification

Two hundred and thirty five yeasts were isolated and identified from the selected five rivers using 26S rRNA gene sequencing. The PCR amplicon size of these yeast isolates for 26S rRNA was between 600 to 650 base pairs (bp) (Figure 6). Of these isolates, 37 were *Candida albicans* that were obtained from the Mooi River (19 isolates), Harts River (9 isolates), Marico River (5 isolates), Crocodile River (3 isolates) and Schoonspruit River (1 isolate) (Table 4). The numbers of the isolates varied but have no quantitative relevance.

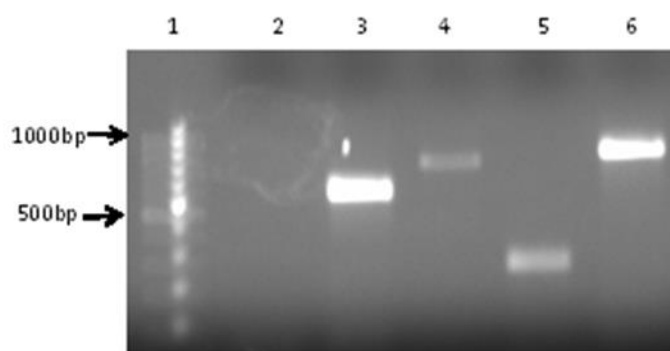


Figure 6. An image of 1% agarose gel indicating amplified gene fragments. Molecular weight marker was used in lane 1. Lane 2 (Non-template control), Lane 3 (26s rRNA; 600bp-650bp), Lane 4 (*CDR1/ CDR2*; 800bp), lane 5 (*FLU1*; 250- 280bp) and lane 5 (*MDR1*; 900bp).

Table 4. Distribution of *Candida albicans* species and efflux resistance genes in NWP Rivers

Sampling site	No of isolates (N)	Efflux resistant genes			
		<i>CDR1</i>	<i>CDR2</i>	<i>FLU1</i>	<i>MDR1</i>
		n/N	n/N	n/N	n/N
Mooi River	19	19/19	19/19	17/19	17/19
Harts River	9	8/9	8/9	6/9	7/9
Marico River	5	5/5	5/5	5/5	3/5
Crocodile River	3	3/3	3/3	3/3	2/3
Schoonspruit River	1	1/1	1/1	1/1	0
Total	37	36	36	32	29

A constructed phylogenetic tree revealed a high gene sequence similarity between clinical sequences from GenBank and environmental isolates of the 26S rRNA gene. A bootstrap confidence of 82% supported the relationship between the isolates. The bootstrap support is based on 1000 replicates (Figure 7).

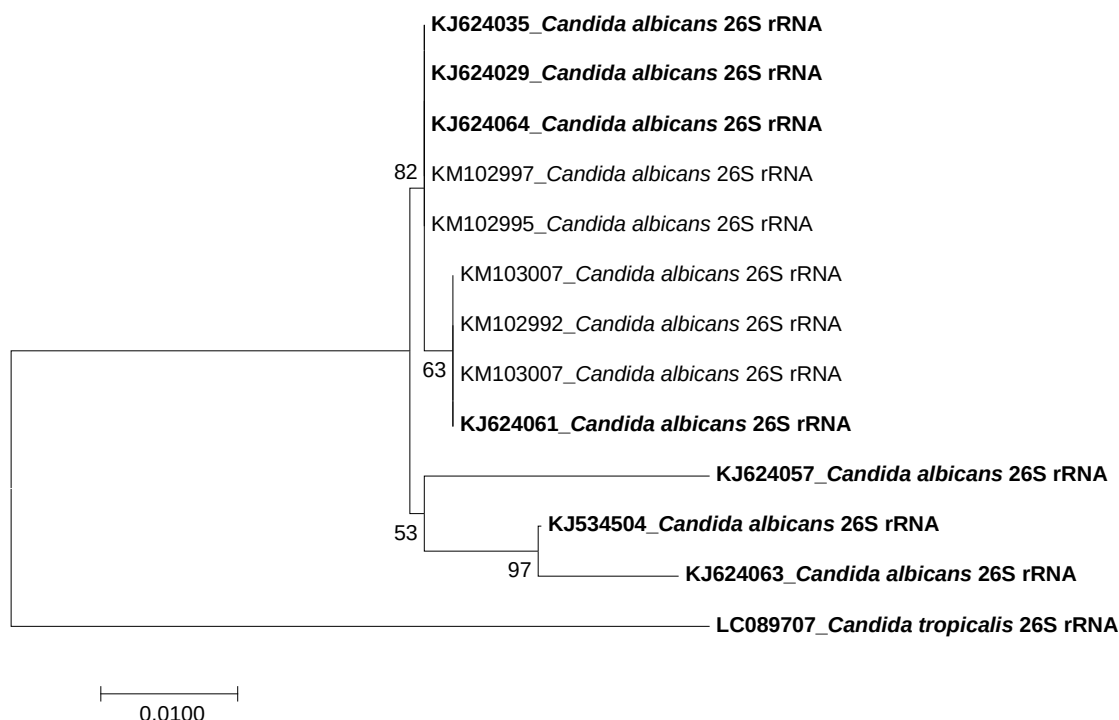


Figure 7. A Neighbour-joining tree showing phylogenetic relationship between *Candida albicans* 26S rRNA gene between environmental and clinical isolates (Bold). A bootstrap test (1000 replicates) was conducted and next to the cluster percentage of trees supporting the cluster is provided.

4.3.2 Antifungal susceptibility

From the identified yeast isolates, susceptibility testing was done on *Candida albicans* using a Kirby Bauer Disk diffusion method. In accordance with the zone breakpoints and interpretative categories for antifungal agents as recommended by CLSI (2009), all *C. albicans* isolates were completely resistant to the following azoles: fluconazole, econazole, ketoconazole, miconazole, and itraconazole. Resistance was also observed to flucytosine, a pyrimidine with a completely different

mechanism of action. However, all the isolates displayed intermediate resistance to nystatin.

4.3.3 Presence of resistant genes in *Candida albicans*

Candida drug resistance (*CDR1* and *CDR2*), multidrug resistance (*MDR1*) and fluconazole resistance (*FLU1*) genes were amplified and detected in all *C. albicans* isolates from the selected rivers (Table 4). PCR amplicon sizes of these resistance genes from gel electrophoresis are shown in Figure 4.1. Efflux pump genes were detected in most ($\geq 60\%$) of the isolates. *C. albicans* isolates had more than one of the genes present. *Candida* drug resistance genes (*CDR1* and *CDR2*) were the most frequently detected genes followed by the fluconazole resistance gene (*FLU1*).

4.3.4 Phylogenetic analysis of the efflux pumps genes

Phylogenetic analysis reveals high gene sequence similarity between clinical sequences from GenBank and environmental isolates from the present study. There was 99-100% bootstrap confidence support for these relations. The bootstrap support is based on 1000 replicates (Figures 8, 9, 10). Primers that were used in the study amplified both *CDR1* and *CDR2*. The clustering between *CDR1/CDR2* genes of study and clinical *CDR1* suggests that the environmental isolates possess the identical *CDR1* gene that is found among clinical strains.

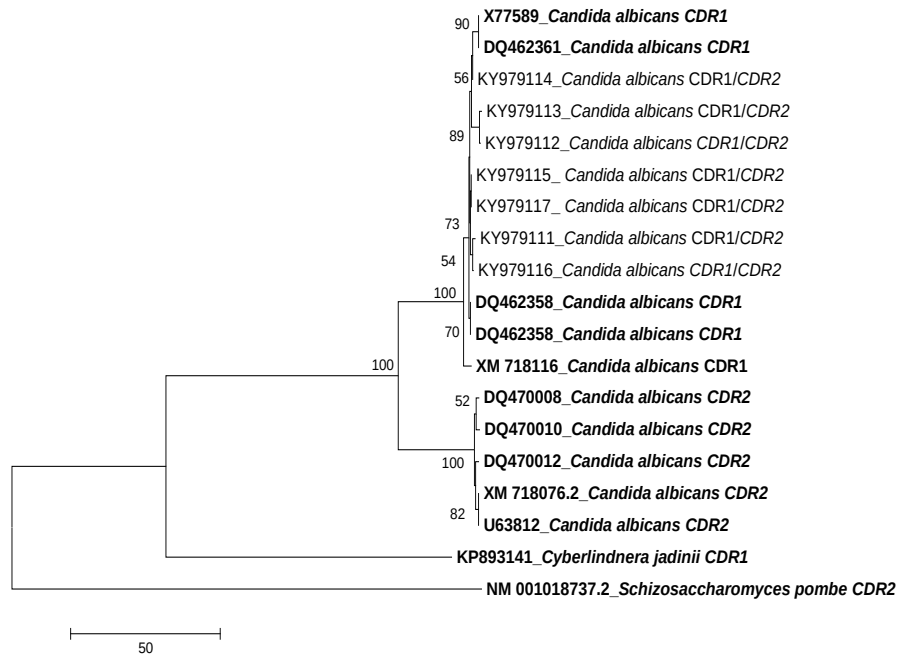
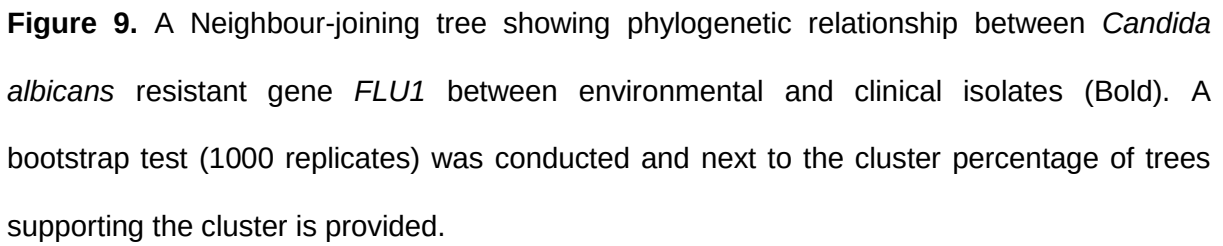


Figure 8. A Neighbour-joining tree showing phylogenetic relationship between *Candida albicans* resistant genes *CDR1* and *CDR2* between environmental and clinical isolates (Bold). A bootstrap test (1000 replicates) was conducted and next to the cluster percentage of trees supporting the cluster is provided.



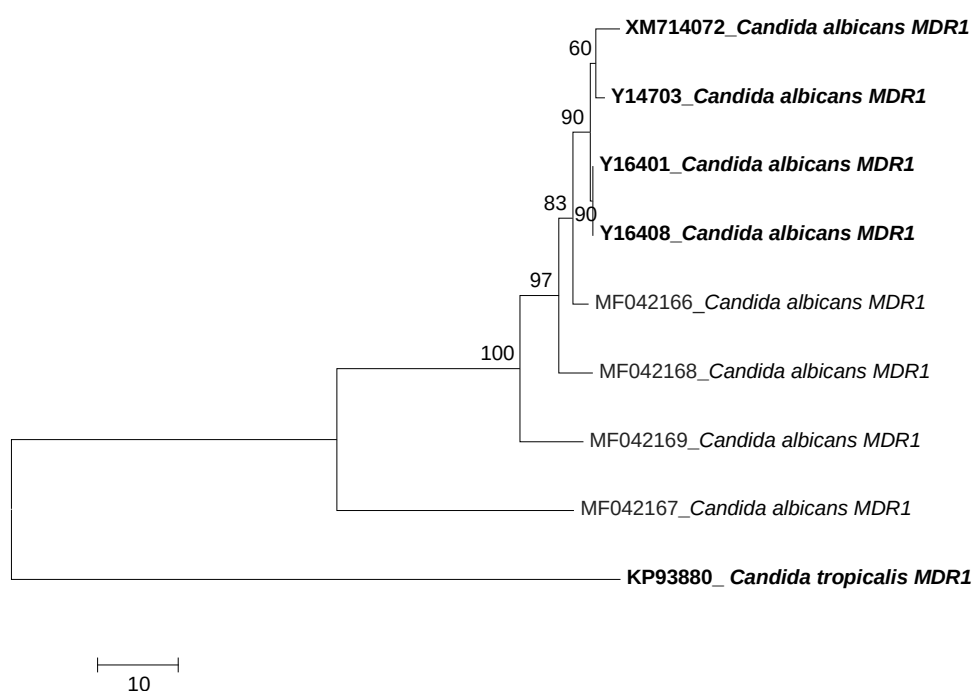


Figure 10. A Neighbour-joining tree showing phylogenetic relationship between *Candida albicans* resistant gene *MDR1* between environmental and clinical isolates (Bold). A bootstrap test (1000 replicates) was conducted and next to the cluster percentage of trees supporting the cluster is provided.

4.4 Discussion

The aim of this study was to determine antifungal susceptibility of *Candida albicans* isolated from NWP Rivers as well as to determine the presence of genes encoding proteins implicated in antifungal drug resistance. A study by Monapathi et al. (2017) has shown that *C. albicans* and other yeast species isolated from two rivers in the NWP were resistant to several clinically relevant antifungal agents that are used to treat yeast infections in humans. There is limited information on the distribution of these pathogenic yeasts as well as the genotype of antifungal resistance. In the

present study, 36 fluconazole resistant *C. albicans* isolates were obtained from environmental water and their efflux pumps mediated resistance was investigated. Resistance genes from these environmental isolates were compared with clinical isolates and similar genes were observed between the two resources. The results from the present study is important when considering that surface water resources in the NWP are used for domestic, mining, recreation and religious purposes as well as agriculture (animal watering and irrigation; NWP-SOER, 2014, Van Wyk et al., 2012, Molale and Bezuidenhout, 2016).

C. albicans, the most common human fungal pathogen with infectious mortality rate of ~40% (Gunsalus et al., 2015) has been isolated from some of NWP water resources (Van Wyk et al., 2012, Monapathi et al., 2017). The presence of *C. albicans* in water has been linked to faecal pollution of water (Cook and Schlitzer, 1981). The origin of this species in the surface water of the NWP could potentially be due to pollution from WWTPs. In many previous studies, WWTPs are implicated in the dissemination of antibiotic resistant bacteria (ARB) and genes (ARGs) to environmental waters (Bouki et al., 2013, Rizzo et al., 2013). The present study is thus contributing to understanding the role of urban WWTPs in the dissemination of the broader resistome to environmental waters. According to DWAF, 2013a, the majority of WWTP in the NWP are not effectively working or poorly managed. The presence of fluconazole resistant yeasts in our rivers confirms this.

In immunocompromised people such as those with immunodeficiency virus infections (HIV), *C. albicans* can cause severe infections when these individuals come in direct contact with the water. This is a concern as the province has a HIV prevalence rate of 13% (Shisana et al., 2014). Many members from urban, peri-

urban and rural communities use the available surface water for recreation and religious purposes that involves direct contact (Coetzee et al., 2016). Infections by antifungal resistant yeast could have devastating effects on the sensitive sector of communities in the North West Province. Similar scenarios could be evident in other provinces or in other developing countries.

In *Candida* infections, antifungal agents especially azoles are used (Rex et al., 1995). A study by Truter and Graz (2015) demonstrated that fluconazole and nystatin are the most commonly prescribed antifungals in South Africa. This drug is also used as the preferred antifungal agent in the prophylactic treatment in HIV positive individuals to prevent *Candida* and *Cryptococcus* infections (Morschhäuser, 2002, Abrantes et al., 2014). The findings of this and a previous study on the resistance to azole antifungals demonstrated general resistance to azoles among environmental *C. albicans* strains. Furthermore, the present study also shows that the genetic elements responsible for the resistance mechanism are similar to what is observed in the clinical settings. This is of great concern. There is a great potential for these genetic elements be selected for and maintained in surface water environments when the selection pressure is maintained. This could be so because fluconazole that is excreted by humans may not be removed by urban WWTPs (Ebele et al., 2017). Such systems had not been designed to perform such functions. These residues could end up in water resources. Furthermore, runoff from agricultural activities may also be responsible for residues of this drug finding its way into the water sources. Such residues, even in very low sub-therapeutic concentrations, would be a sufficient selective pressure for the maintenance of antifungal resistant yeast strains and associated resistance genes. Similar

observations had been made in clinical scenarios where prolonged overuse of these drugs was responsible for the selection of drug resistant yeast strains (Luque et al., 2009, Abrantes et al., 2014). Fluconazole resistance is a major problem as the drug has been routinely administered to treat candidiasis in health care facilities in the African continent for an extended period (Powderly, 1999, Abrantes et al., 2014.).

In the present study, susceptibility test screening was performed on *C. albicans* isolated from NWP Rivers. The various yeast species from the different river systems presented the same susceptibility pattern to the antifungal drugs. They were resistant to azoles and flucytosine and also showed intermediate resistance to nystatin. In the treatment and prophylaxis of oro-oesophageal candidiasis in the early 1990s, fluconazole became the antifungal of choice (Maenza et al., 1997). However, in the years following the introduction of fluconazole, resistance was reported in 41% of the patients (Canuto and Roderio, 2002). The data reported in this study on *C. albicans* conforms to previously published data regarding its susceptibility to fluconazole (Hazen et al., 2003, Pfaller and Diekema, 2004). Multiple antifungal agents can be substrates for efflux mediated ABC transporters and their expression may lead to cross resistance to different drugs (Ramage et al., 2002). This could explain *C. albicans* resistance to various antifungal agents (White, 1997).

Development of resistance to fluconazole by *C. albicans* is a genetically generated micro-evolutionary change during antimycotic therapy (Morschhäuser, 2016). In the present study, the fluconazole resistance was linked to the presence of specific efflux pumps that could be intrinsic or acquired from external sources (Li and Nikaido, 2004). In yeast genomes there are more than thirty reputed efflux pumps

mediated genes. In the present study multidrug associated resistance (*MDR1* and *FLU1*) and the candida drug resistance (*CDR1* and *CDR2*) genes were determined in fluconazole resistant *Candida albicans*. These genes were present in the genomes all isolates from geographically different sampling sites. The expression of these genes could either be individually or simultaneous mediated (Franz et al., 1999, Perea et al., 2001).

Efflux genes were the cause for resistance to fluconazole by clinical isolates from HIV infected patients (White, 1997, Lopez- Ribot et al., 1998). The present study shows that the same genes were also present in *C. albicans* isolated from environmental water resources. A high bootstrap confidence values indicate close phylogenetic relationships of the environmental isolates to the genes of clinical ones suggesting that the isolates could be from clinical origin or that the genes were disseminated to the environmental isolates (Ogunseitán, 2005, Hall, 2013, Jones et al., 2004, Holmes et al., 2006). There are limited studies on the sequences of resistance genes in yeast and no sequences from isolates of environmental origin could be found in GenBank. Two scenarios could be speculated on for the detection of these phylogenetic poorly distinguishable gene sequences: (i) the genes were disseminated from clinical strains to environmental ones and that these are now wide spread in the aquatic environment of the North West Province (ii) that the strains isolated were from clinical sources and landed in the water sources, due to pollution. Whichever of these scenarios hold true, it is with apprehension that the antifungal resistance patterns and presence of these genes in the surface waters of the North West Province are noted.

Most of the *C. albicans* in the present study were isolated from the Mooi River. The river is used for irrigation and recreational activities such as swimming and angling (le Roux, 2005, Van Der Walt et al., 2002) as well as for religious purposes (Molale and Bezuidenhout, 2016). The other rivers are used for similar purposes. Contamination of these rivers with the pathogenic/opportunistic yeast such as *C. albicans* and direct exposure of these could result in major health problems especially the immunocompromised.

Globally, only a small percentage of rivers are minimally affected by anthropogenic activities. These are rivers in remote areas with low populations (Vörösmarty et al., 2010). To minimize environmental water contamination, proper and effective management and extensive investments in infrastructure should be used on WWTPs. Public participation and education for the community on the proper use and effects of domestic and agricultural discharges on the river water is required. Furthermore, environmental laws and regulations should be enforced on chemical and agricultural industries with discharges that affect the river water quality (Enderlein et al., 1997).

4.5 Conclusion

Antimicrobial resistance (AMR) is a growing worldwide health risk and major threat to public health. In the present study, antifungal resistance patterns were demonstrated for a human pathogenic yeast species, *C. albicans* that was isolated from four NWP rivers. All the isolates were resistant to fluconazole and other azole containing antifungal agents. This is a cause for concern because fluconazole is the most prescribed drug in South Africa. It is used as part of the prophylactic treatment of

immunocompromised individuals such as those that are HIV positive. Overexpression of efflux pumps has been correlated with antifungal resistance in *C. albicans*. In the current study, ABC transporter genes (*CDR1* and *CDR2*) and major facilitator gene (*FLU1* and *MDR1*) were present in all the fluconazole resistant isolates. It is of great concern that there are more than one of these efflux mechanisms in *C. albicans* environmental isolates. This could result in cross-resistance to various drugs, resulting in multiple antifungal resistance. The presence of resistance genes in clinical isolates is a public health concern and requires further investigation since it is known that the environmental water systems are used for direct contact activities. The results from the present study calls for investigation into the mechanism of antifungal resistance of yeasts from environmental resources using carefully designed experiments to determine the expression profiles of these genes when present in environmental and clinical isolates. The study on efflux pump resistance mechanisms could help in the development of new strategies to combat the resistance problem.

CHAPTER 5

Yeast and antifungal drugs levels from polluted surface water: perspective on antifungal resistant yeast

5.1. Introduction

Yeasts have been isolated from freshwater environments and have been found in high abundance in polluted waters (Medeiros et al., 2012, Van Wyk et al., 2012, Monapathi et al., 2017). Some of the isolated yeasts were characterized as pathogenic with potential to cause serious infections (Gould, 2012, Whaley et al., 2016). The quality of surface water is compromised by discharges from industries, agricultural run-off and poorly treated sewage, particularly when hospital wastewater is taken into account (Alrhoun, 2014). This is a cause for concern as surface water resources are used for recreational, religious and agriculture (animal watering and irrigation) purposes (Van Wyk 2012, Molale and Bezuidenhout 2016). The occurrence of pathogenic or opportunistic yeasts in surface water is a public health threat. People that come in direct contact with such polluted water especially immunocompromised individuals, could be infected by these species.

Studies from aquatic environments have reported on the isolation of yeasts using culture dependent techniques (Medeiros et al., 2008, Brandão et al., 2010:2011, Ayanbimpe et al., 2012, Krause et al., 2013, Van Wyk et al., 2012, Monapathi et al., 2017:2018). Some studies have applied culture independent methods such as environmental DNA (eDNA) extraction to show the presence of some microbial species and to detect faecal pollution (Caldwell et al., 2011, Jerde et al., 2011,

Nielsen et al., 2007). eDNA analysis is rapid, efficient and most useful in cases where traditional sampling strategies may prove inadequate (Jerde et al., 2013, Barnes and Turner, 2016). It is cost effective and could be standardized for survey and to monitor diversity of freshwater organisms, terrestrial sediments and ice cores to characterize their microbial communities (Hofreiter et al., 2003, Willerslev et al., 2007, Thomsen et al., 2012, Biggs et al., 2015).

Analysis of eDNA could be used to indirectly infer the presence of a species (Jerde et al., 2013). In such approaches, real time quantitative PCR (qPCR) is used to amplify and simultaneously quantify targeted DNA sequences of individual species (Thomsen et al., 2012, Wilcox et al., 2013). The number of gene copies or relative number of gene copies in a complex DNA sample is determined. The amplified gene copy number from bulk DNA reflects the relative abundance of DNA of the specific organism in the complex eDNA sample (Furet et al., 2004). The techniques have been used to quantify microbial composition in fermented foods and wine (Hierro et al., 2006, Park et al., 2009, Makino et al., 2010, Soares-Santos et al., 2017:2018).

Pharmaceutical compounds have been found in the environment at low normally subtherapeutic concentrations (Schwarzenbach et al., 2007, Kümmerer, 2009). Their sources could be hospitals, landfills, private households and agriculture (Kümmerer, 2009). Antifungal agents, used in human, veterinary pharmaceuticals and agriculture (Heusinkveld et al., 2013) are amongst these pharmaceuticals. Various antifungal agents that are azole derivatives such as fluconazole, ketoconazole, clotrimazole, econazole, itraconazole and miconazole are mostly used in human medicine to treat yeast infections (Bondaryk et al., 2013). A large proportion of the active compounds

are not metabolized in the body and are excreted through the urine and skin during washing and find their way into wastewater (Kahle et al., 2008, Kim et al., 2009). Some of antifungal drugs are also used in animal husbandry as growth promoters (Kümmerer, 2009). Another major use of antifungals is in crop protection, horticulture and prevention of post-harvest losses (Jampilek, 2016, Dalhoff, 2017). These substances may thus be present in water downstream from where various agricultural activities occur. Various pharmaceutical products, including antifungal agents are thus continuously disposed of into the environment. They are discharged into the rivers from treated wastewater, sewage sludge that was applied to soil as fertilizer (Murdoch, 2015).

In HIV treatment regimes, antifungal agents are used to treat related mycotic infections but are also used as prophylactic prevention of mycotic infections (Moges et al., 2016). In South Africa, 3.9 million people are living with HIV (Statistics SA, 2017). This is a considerable component of the population. Widespread and continued exposure of yeasts to antifungal drugs has resulted in antifungal resistance (Morschhäuser, 2016). The objectives of the present study were to (i) gain some perspective on the levels of yeasts in water based on qPCR quantification of 26S rRNA gene copy numbers and (ii) determine the presence of antifungal drugs in an urban river in the NWP.

5.2. Materials and methods

5.2.1. Sampling area and procedure

Water samples were collected from 8 sampling sites in Mooi River, NWP and 2 from the Wonderfonteinspruit River a tributary of the Mooi River (Figure 11). Water samples were collected aseptically in 1L sterile bottles using the direct and dip sampling technique. The samples were protected from light and transported on ice to the laboratory for analysis within 8 hrs. Sampling was conducted in the wet season in 2017 and 2018.

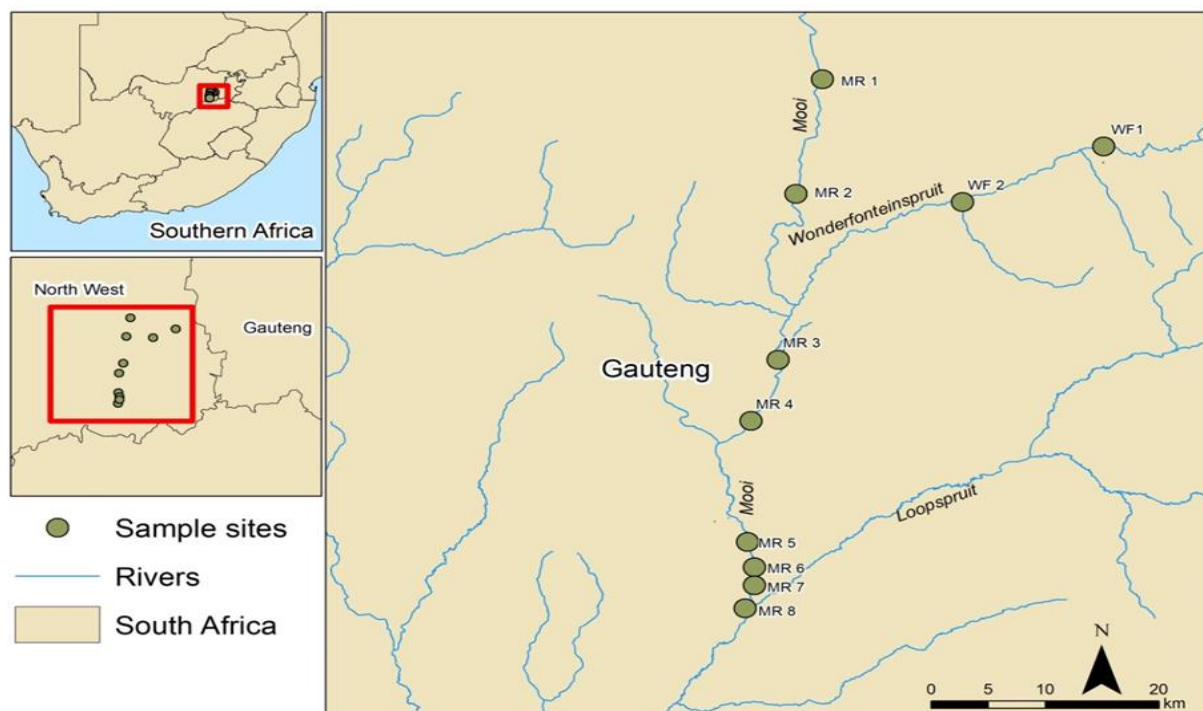


Figure 11. Map showing the sampling sites from the Mooi River and Wonderfortein Spruit River tributary into the Mooi River. (MR=Mooi River, WF= Wonderfonteinspruit River).

5.2.2. eDNA extraction

Samples were analyzed in triplicate by membrane filtration through a 0.45 µm membrane filter to recover yeasts (Van Wyk et al., 2012). Filter holders, forceps that came into contact with the eDNA samples were sterilized between each sample by soaking in 70% ethanol. Filters from 1L water samples from each sampling were used for extraction. eDNA was extracted using the DNeasy PowerWater® kit (Qiagen, Germany) with minor modifications: PW1 (Lysis buffer) and PW3 were incubated for 20-30 min in a water bath at 60°C. Ten microliters of Proteinase K (10µg/ml) and 5µL of Rnase (10µg/ml) were added and briefly (1 min) vortexed. This was followed by incubation at 60°C for 30 min and subsequently vortexed. The protocol of this kit (DNeasy PowerWater® kit, Qiagen, Germany) was followed from step 8 onwards. A NanoDrop TM 1000 spectrophotometer (Thermo Scientific, USA) (NanoDrop, 2007) was used to measure DNA concentrations and 260/280 ratios to determine the purity of the DNA.

5.2.3. Specificity of PCR assays

A convectional PCR was performed to amplify eDNA using the 26S rRNA gene primers. Universal primers YEASTF (5'-GAGTCGAGTTGTTTGGGAATGC-3') and YEASTR (5'-TCTCTTTCCAAAGTTCTTTTCATCTTT-3') specially designed (Hierro et al., 2006) for yeasts amplification were used. These yeast primers contain conserved sequences of the variable D1/D2 domains of the 26S rRNA gene and have an amplicon fragment size of 124 bp. PCR reagents were prepared to a final volume of 25 µL with (i) 12.5 µL double strength PCR Master Mix (0.05 U/µL Taq polymerase, 4 mM MgCl₂ and 0.4 mM dNTPs; Fermentas Life Sciences (USA), (ii) 1.5 µL MgCl₂, (1.5 mM), (iv) 3.0 µL primer mix and (v) 6 µL nuclease free water

(Fermentas Life Sciences, USA) (iii) 2 µL genomic template DNA (± 20 ng). DNA of *Candida glabrata* (accession number-KY778701) identified from the 26S rRNA gene sequencing was used as a positive control. The PCR cycling conditions were performed according to Monapathi et al. (2017). Confirmation of the 26S rRNA PCR amplicons was achieved by agarose gel electrophoresis.

5.2.4. Real-time RT-PCR (q-PCR) analysis

The experimental design, procedures and data analyses followed the MIQE Guidelines when applicable (Bustin et al., 2009). Reaction mixtures were set up on a MicroAmp Optical 96-Well Reaction Plate with Barcode (Applied Biosystems, USA).

5.2.4.1. qPCR reactions

Quantitative PCR reactions were performed on Bio-Rad CFX96™ system using SsoFast™ EvaGreen Supermix kit (Bio-Rad). PCR conditions were performed in 20 µL volumes containing 10 µL of SsoFast EvaGreen supermix, 1 µL of each universal 26S rRNA (YEASTF 5'-Sequences-3' and YEASTR 5'-Sequences-3') primers, 2 µL of eDNA template, and 6 µL of H₂O. PCR conditions comprised of 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 5 seconds. Melt curve analysis was implemented on qPCR assays in order to verify specificity. Melting curve analysis was done to confirm primers-specific products amplification. The curve was generated from 65 to 95°C with increments of 0.5°C/cycle with continuous collection of fluorescence data.

5.2.4.2. Standard curve

Sensitivity of the qPCR was determined by constructing a standard curve using serial dilutions of known concentrations from pure genomic DNA template of *Candida glabrata* (accession number-KY778701). A starting mass (20 ng) of pure yeasts DNA was serially diluted 10-fold in triplicate. Bio-Rad CFX96™ instrument automatically calculated cycle threshold (Ct) values, linear regression model ($10^{-1/\text{slope}}$) and efficiencies (%E).

5.2.4.3. Determination of yeast 26S rRNA gene copy number in the environmental water

eDNA extracted from the water was included in the quantitative PCR assay. Ct values from the assays were compared to those from a standard curve to determine the mass of the environmental DNA at their respective Ct values. Copy numbers were derived from DNA mass (Park et al., 2009).

5.2.5. Antifungal drugs: quantification and screening

5.2.5.1. Chemicals and reagents

Fluconazole analytical reference standard and deuterated fluconazole (d-4) internal standard were obtained from US Pharmacopeial Convention purchased from Stargate Scientific (Johannesburg, South Africa), and Toronto Research Chemicals (Toronto, Canada), respectively. Both standards were $\geq 99\%$ purity. Stock solutions (1 mg/mL) of the standards were prepared gravimetrically in methanol and stored at -20°C in amber vials. Spectrometry grade acetonitrile and methanol were obtained from Honeywell Burdick and Jackson (Anatech Analytical Technology, Johannesburg, South Africa), and Acetic acid (98% purity) was obtained from Fluka

(Sigma, Germany). Double distilled, nanopure, water was obtained from an ELGA water purification system. Oasis HLB-L (hydrophilic–lipophilic balance low) solid phase extraction (SPE) disks (47 mm) and pre-filters were obtained from Atlantic (Horizon Technology, Salem, USA).

5.2.5.2. Environmental sample extraction

An internal standard (fluconazole d-4) was added to the water samples in the laboratory and stored at 4°C until extraction. The SPE-DEX (Horizon, USA) automated solid phase extraction system was used to extract fluconazole from 1 L of the sampled water. The extraction method was based on that described by Ferrer and Thurman (2012). Samples were extracted using Oasis HLB-L disks topped with 5 µm and 1 µm pre-filters. The disks were conditioned with methanol twice with a 30 seconds soak time, and a 15 seconds air dry period between steps. This was followed by a 10 seconds methanol conditioning with a subsequent two second air dry period. The disk was further conditioned twice with nanopure water for 10 seconds with a two second dry time between conditioning steps. The water sample was immediately passed through the SPE system followed by a 15 minutes air dry period. The fluconazole was eluted with three methanol cycles, the methanol soaked on the disk for three minutes followed by 20 seconds air dry, and the final methanol elute soaked for one minute with a one minute air dry. The eluate was concentrated to near dryness under a gentle stream of nitrogen gas at 40°C. The sample was reconstituted in 500 µL methanol.

5.2.5.3. Matrix-matched-calibration

An external matrix-matched-calibration curve was used to account for matrix effects during quantification. To attempt to mimic naturally occurring water in the matrix-matched calibrations, double distilled water was supplemented with 0.7 mM NaHCO_3 , 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 75 μM KCl to create artificial freshwater (ISO, 2012). The ISO water was supplemented with 5% ISO freshwater that had had 25 mature freshwater snails (*Bulinus tropicus*) housed for at least 24 h. This was done in an attempt to simulate at least some form of organic content. This matrix was extracted identically to the samples.

Concentrations for the calibration curve were determined based on the expected environmental levels of fluconazole in the samples and the performance of the instrument. Samples were concentrated 2 000 times during extraction. The calibration range included: 0, 25, 50, 75, 100, 200, 300, 400, 500 $\mu\text{g/L}$. The matrix-matched extracts were spiked with fluconazole at the calibration range concentrations and 20 $\mu\text{g/L}$ fluconazole-d4. Dilutions were not serially prepared but originated from different stocks. These standards were analysed in triplicate to assess the reportable range (Westgard, 2008). They were injected in order of increasing concentration, with blank injections between batches to monitor carry-over.

5.2.5.4. LC/MS targeted analysis

Sample extracts were analysed by ultra-high pressure liquid chromatography (UPLC, Agilent 1290 series) coupled to a quadrupole time of flight mass spectrometer (Q-TOF/MS, Agilent G6540A). After injection of 1 μ L sample the fluconazole was resolved on a Poroshell 120 Bonus-RP column (Agilent, 2.1 x 100 mm, 2.7 μ m) that was kept at 25°C with mobile phase (A) water and (B) acetonitrile, both containing 0.1% acetic acid. The UHPLC was run at a constant flow rate of 0.7 mL/min. The chromatographic gradient was as follows: 5% B 4.5 min, 10% B 7 min, 50% B 11 min, 100% B 12.2 min. The target compounds fluconazole (307.0968 [M+H]) and fluconazole-d4 (311.1236 [M+H]) had retention times of 6.38 and 6.33 min respectively. The ionisation source was an Agilent Jet Stream electrospray ionisation and performed in positive ionisation.

Source settings were optimised as follows: drying gas temperature 250°C, drying gas flow 8 L/min, nebuliser pressure 35 psi, sheath gas temperature 300°C, sheath gas flow 10 L/min, VCap 3 000 V, nozzle voltage 0 V, fragmentor 130 V, skimmer 48 V, and OCT RF Vpp 750 V.

5.2.5.5. LC/MS screening

The same system as mentioned above was used for the screening of additional antifungal agents. The list investigated was: econazole, flucytosine, ketoconazole, miconazole, metronidazole and nystatin. The QTOF was set to scan from 50 to 950 m/z and the instrument was set to extended dynamic range (2 GHz). Software used was MassHunter Data Acquisition (version B.05.00), MassHunter Qualitative Analysis (version B.05.00). Mass axis calibration of QTOF was performed daily for

positive ionisation with tuning mixes (G1969-85000, Agilent). A reference solution with masses of 121.050873 [M+H] and 922.009798 [M+H] were constantly infused as accurate mass references. The accurate masses of the compounds were used to search for their presence, but their exact concentrations were not determined at this time.

5.2.5.6. Precision and accuracy

Precision (repeatability, in terms of % RSD) and accuracy (percentage recoveries) were estimated by recovery experiments, each one analysed in triplicate. Accuracy was determined as the recovery of the spiked analyte relative to the internal standard. Average recoveries were 85%. Precision was calculated using $\% \text{ RSD} = (\text{SDEV of QCs} / \text{mean of QCs}) \times 100$. The method was found to also have satisfactory precision, with RSDs at 7%.

5.2.5.7. Linearity

Linearity of the calibration curve was assessed by determining the R^2 value. Good linearity is indicated with R^2 as close to 1 as possible (at least 0.9) (Miller and Miller, 2010). The linearity of the calibration curve for this analysis is $R^2 = 0.9972$ (Figure 12).

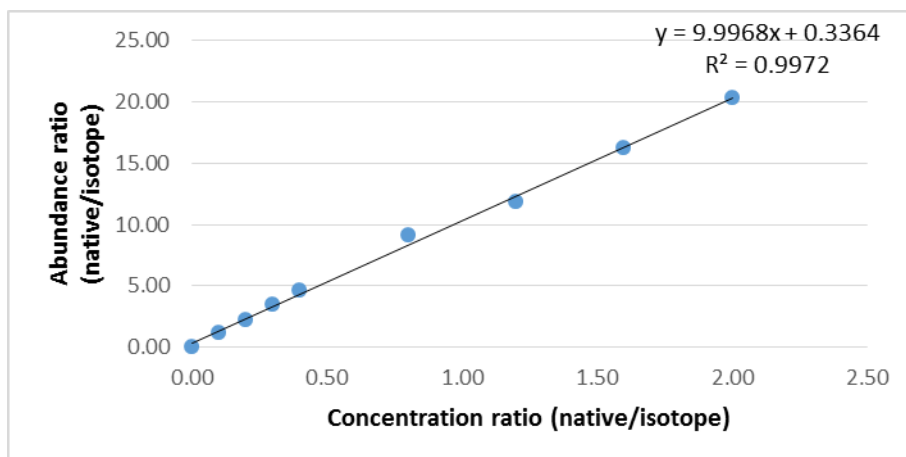


Figure 12. The calibration curve used in the quantification of the samples. This is based on the ratio between the native standard, fluconazole, and the internal standard (fluconazole-d4 isotope).

5.2.5.8. Limit of detection (LOD)/Limit of quantification (LOQ)

Sensitivity of an analytical method is defined as the increased response of the analyte linear to the analyte concentration (Whitmire et al., 2011). This is displayed with a calibration curve and the slope of the calibration curve (Figure 12). By using linear regression statistics the uncertainties of the calibration curve can be used to calculate LOD and LOQ for the method from the external matrix matched calibration curve. By use of the $y=mx+c$ model, LOD is calculated by $3 \cdot S_a/b$ and LOQ by $10 \cdot S_a/b$; where S_a is the SD of the intercept (abundance) and b is the slope of the calibration curve (Schoeman et al., 2015). The LOD for this analysis was 30 $\mu\text{g/L}$ and the LOQ 101 $\mu\text{g/L}$. The concentrations of the extracts above the LOQ are valid.

Data analysis:

The concentration of fluconazole in samples was calculated by using this formula:

$$X_{\text{fluconazole}} = ((\text{native/stable isotope}) - c) / m \times \text{ISO conc}$$

where:

$X_{\text{fluconazole}}$ = calculated analyte concentration

native = native abundance

stable isotope = stable isotope abundance

c = calibration curve y-intercept

m = slope of calibration curve

ISO conc = stable isotope concentration

5.2.6 Statistical Analyses

One-way ANOVA (ANALYSIS Of VARIance) with post-hoc Tukey HSD (Honestly Significant Difference) Test for equal sample sizes was used to verify significant differences in copy numbers in eDNA between the two sampling periods.

5.3 Results and discussions

5.3.1 PCR product integrity

The quantity and quality of the DNA isolated directly from water (eDNA) was determined using Nanodrop measurements. DNA concentrations ranged from 17 to 321 ng/μL and the A_{260}/A_{280} quality scores ranged between 1.7 and 2.1. Pure DNA (~1.8) (NanoDrop, 2007) was measured in some of the DNA samples. Successes of the 26S rRNA PCR reactions were determined by agarose gel electrophoresis.

Amplicons (124 bp) were observed for all eDNA from both sampling periods (Figure 13). The primers and approach used in the present study was similar to that of Hierro et al. (2006). These authors (Hierro et al., 2006) designed the primers and also tested whether the PCR was successful using agarose gel electrophoresis. Their interest was to enumerate total yeasts in wine. The authors also found the same expected amplicon sizes of the PCR product. In the present study, the PCR results indicated the presence of yeast 26S rRNA genes in water. In previous studies (Van Wyk et al., 2012, Monapathi et al., 2017) yeasts were isolated from the same sampling sites. Soares-Santos et al. (2018) equated copy numbers to levels of yeast in wine. Similar assumptions could thus be made for the copy numbers of yeast barcode genes in aquatic ecosystems.

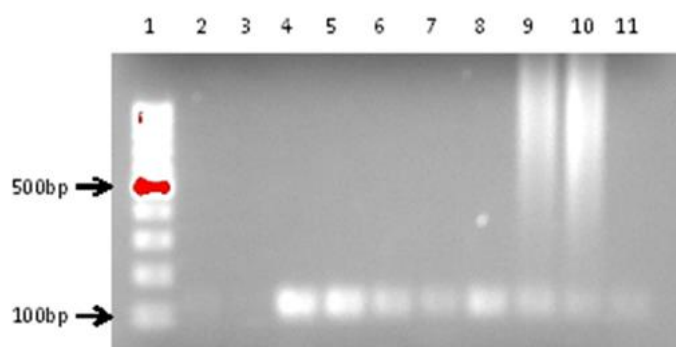


Figure 13. An image of 1% agarose gel indicating amplified gene fragments. Lane 1= molecular weight marker, lane 2= non-template control, lane 3= negative control (Bacterial DNA), lane 4= positive control (Pure yeast DNA; 26S rRNA PCR gene fragments), lane 5-11= Environmental DNA.

5.3.2. qPCR standard and sensitivity

The target sequences length was suitable for qPCR (80–150bp) for specific and efficient amplification (Bustin and Huggett, 2017). The constructed standard curve met the requirements stipulated in MIQE Guidelines (Bustin et al., 2009). The amplification plot generated a slope of -3.23 with a correlation coefficient of 0.99 (Figure 14). The assay was linear over 5 orders of magnitude. These results indicated that it is only possible to quantify samples of unknown concentration accurately within this range of concentrations. The melting curve obtained for the pure DNA isolates generated a T_m of 79.3°C (Figure 15). A single peak formed. This demonstrated that the primers bound only to the specific target product at pre-determined temperature (Figure 15). No dimers of oligonucleotide primers or non-specific products were present to cause non-specific amplification (Bustin and Huggett, 2017).

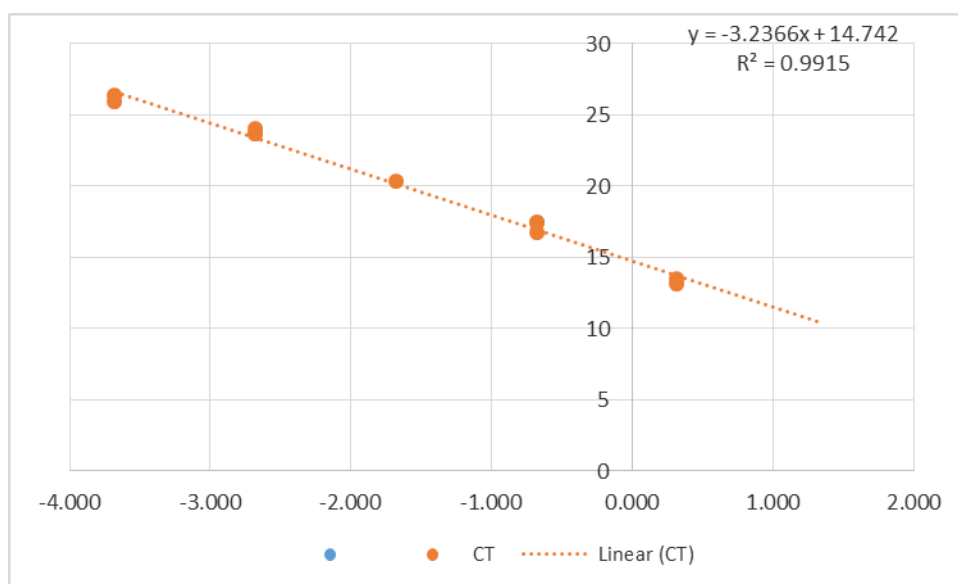


Figure 14. Standard curve obtained from serially diluted pure genomic DNA. Ct values are the average of three repetitions.

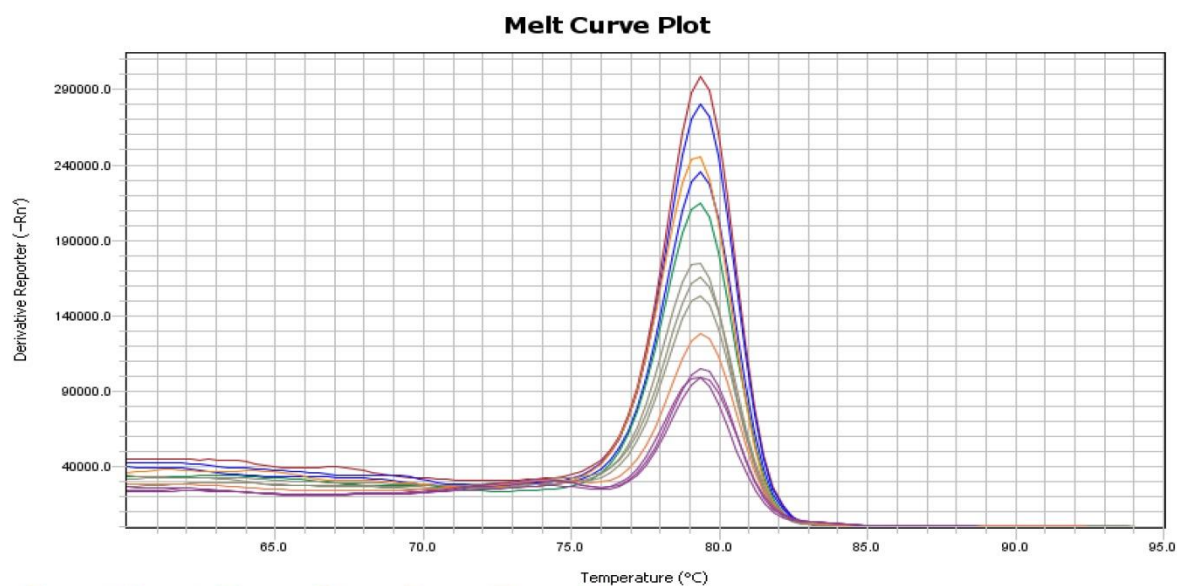


Figure 15. Melt curve obtained from serially diluted pure genomic DNA with T_m of $\sim 79.3^\circ\text{C}$.

5.3.3. 26S rRNA gene copy numbers

Park et al. (2009) conducted a quantitative PCR study to enumerate total bacteria, archaea and yeast populations in fermented foods. The author used the same target gene (26S rRNA) with same primer pair used in the present study to amplify yeast DNA. In their findings, 1 nanogram of DNA contained 2.54×10^4 to 2.43×10^5 copies of yeast DNA. Thus with reference to the study of Park et al. (2009), 1 nanogram of environmental DNA isolated in the present study contained 2 to 18477 copies of yeast DNA. The averages of yeast DNA copy numbers are shown in Figure 16. These copy numbers infer relative abundance of yeasts in water.

High levels of yeasts (26S rRNA gene copy numbers) observed in a study by Park et al. (2009) is expected. Yeasts are associated with the fermentation process. In the present study, the 26S rRNA gene copy numbers were low. This is understandable since yeasts in aquatic ecosystems will be and thus in low proportion in the eDNA.

Environmental DNA comprises a large quantity of genetic material from viruses, whole microbial cells or shed from multicellular organisms (Torti et al., 2015) thus yeasts would only make a small fraction.

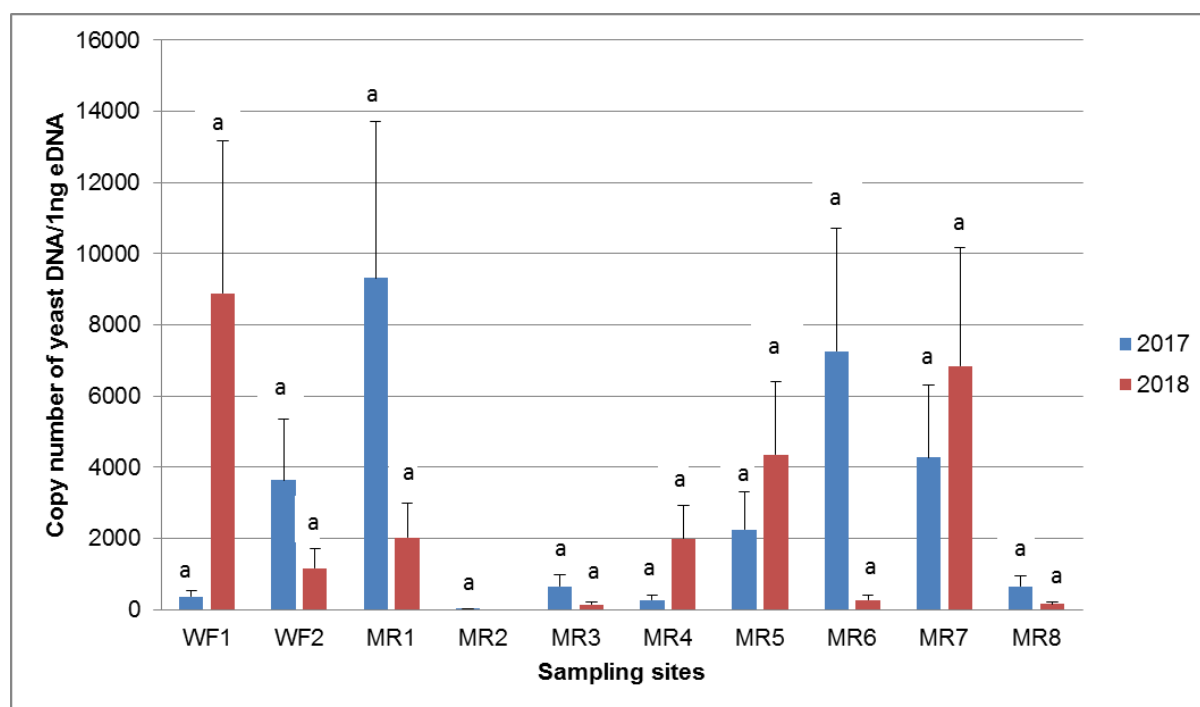


Figure 16. Averages of yeast DNA copy numbers in Mooi and Wonderfortein Spruit River

Quantitative PCR studies have also been conducted on wine products to quantify yeasts (Hierro et al., 2006, Soares-Santos et al., 2018). In a study by Hierro et al. (2006), total yeasts ranged between 1.7×10^2 to 3.5×10^7 cells/mL for wine samples. The author used conventional qPCR relying on DNA as a template similar to the present study. Soares-Santos et al. (2018) determined that ranges between 10^2 and 10^7 cells/mL of quantifiable yeast cells could be determined by Cells-qPCR. In this approach DNA was not extracted and intact cells were used in the PCR.

Copy numbers in the current study fluctuated between sites and sampling periods. However, no significant differences were observed between the sampling points and periods (Figure 16). In some sampling sites (MR2, MR3, MR4 and MR8), comparatively low copy numbers were seen. According to Schultz and Lance (2015) species detection using the eDNA extraction method is subject to chance with detection probability. Yeast levels were higher in 2017 for sites WF1, MR 6 and MR7 compared to the other sampling sites. However, in 2018, the WF2, MR6 and MR7 had higher levels. These results indicate that impacts at Wonderfonteinspruit River sites and two of the Mooi River sites are such that elevated levels of yeast occur at these sites. Low viable yeasts levels (107- 147 CFU/L) were reported previously from the same sampling sites (Monapathi et al., 2017).

Nagahama. (2006), Brandão et al. (2010) and Medeiros et al. (2012), associated yeasts in aquatic environments with organic pollution. The WF1 and WF2 site could be receiving pollutants from informal settlements, urban treated sewage effluent and runoff and mining effluents from Carletonville in the Gauteng Province (Van Aardt and Erdman, 2004). Sampling sites MR6 and MR7 are situated downstream of the town of Potchefstroom. The area is characterised by a high number of property developments. Leaking sewage could decant into the river from storm water systems. High counts of indicator and heterotrophic bacteria were isolated from the same sampling sites and MR8 (Jordaan and Bezuidenhout, 2016). The latter sampling site receives wastewater effluent from Potchefstroom sewage works (Jordaan and Bezuidenhout, 2016). However, in the present study, low levels of yeasts were enumerated at sampling site MR8.

An extensive search in various databases could not yield any results on the quantification of yeasts in environmental water, using qPCR. However, studies using qPCR for wine and fermented food products demonstrated that the approach is rapid and precise and a useful tool to determine the risk of spoilage (Hierro et al., 2006, Park et al., 2009, Soares-Santos et al., 2018).

5.3.4. Antifungal drugs in surface water

Quantifiable levels for fluconazole (178 ng/mL to 271 ng/mL) were measured in Wonderfonteinspruit River sampling sites in both sampling periods (Table 5). However, fluconazole levels were below detection levels in all Mooi River sampling sites. Water samples were also screened for various other antifungal agents (Table 5). This was a qualitative approach and indicated the presence/absence of the drug per sampling site. From these results it is evident that three of the agents most frequently present were metronidazole, flucytosine and ketoconazole. Econazole and nystatin were not detected at any of the sites. The Wonderfonteinspruit River flows through Carletonville, a town characterized by rapid urbanization and gold mining activities (Van Aardt and Erdman, 2004). Both these activities result in an influx of people from various parts of the province and the country. This could also mean that potentially large number of HIV positive individuals, on fluconazole treatment could also migrate towards this and surrounding towns.

Finding these drugs in surface water in the North West Province should not come as a surprise. It could be as a result of their therapeutic, prophylactic and agricultural use (Mateo et al., 2013, Singer et al., 2016) Due to its safety profile and bioavailability, fluconazole is mostly used in South Africa to treat HIV positive patient

(Morschhäuser, 2002, Truter and Graz, 2015) as well as to prevent mycotic infections. High prevalence of HIV in the country, suggests that the use of fluconazole for prophylactic and therapeutic purposes is also proportionally high. Fluconazole is not completely metabolized and ends in the sewage. This is transported to WWTPs and in the effluent that is transported to rivers systems (Kim et al. 2007, Kahle et al., 2008, Kim et al., 2009). In WWTPs that work efficiently, pharmaceutical products are only partially removed (Kümmerer, 2009). When WWTPs are working poorly the chance of the antifungal agents landing in receiving river systems is increased (Yang et al., 2014). Furthermore, antifungal drugs are used in agriculture to protect grain crops from fungal diseases (Jampilek, 2016, Dalhoff, 2017). Run-off from these systems could result in decanting these drugs into surface water ecosystems.

Table 5. Quantitative and qualitative analysis of antifungal agents in Wonderfonteinpruit (WF) and Mooi Rivers (MR) sampling sites (√= detected; nd= not detected; LOD= Level of detection). Qualitative values are proportions of antifungal agents present in the water.

Method of analysis	Sampling times	Antifungal agents	Sampling sites								
			WF1	WF2	MR1	MR3	MR4	MR5	MR6	MR7	MR8
Quantitative (ng/mL)	2017	Fluconazole	204.4	178.0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2018		189.4	271.1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Qualitative	2018	Flucytosine	√	√	nd	nd	nd	nd	nd	√	√
		Metronidazole	√	√	√	√	√	nd	nd	nd	nd
		Econazole	nd	nd	nd	nd	nd	nd	nd	nd	nd
		Ketoconazole	nd	√	nd	nd	√	nd	nd	√	√
		Miconazole	√	nd	nd	nd	nd	nd	nd	nd	nd
		Nystatin	nd	nd	nd	nd	nd	nd	nd	nd	nd

Finding measureable levels of the yeast marker (26S rRNA genes) and antifungal agents in water resources is a concern as some of the yeasts could be pathogenic. Continuous exposure to these antifungal agents could result in antifungal resistance selection and maintenance (Morschhäuser, 2016). Antifungal resistance has been reported in yeasts from aquatic environments (Medeiros et al., 2008, Brandão et al., 2010, Brilhante et al., 2016, Monapathi et al., 2017). In the current study area (Mooi and Wonderfoteinspruit River, pathogenic yeasts: *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, *C. pseudolambica*, *C. tropicalis*, *Cyberlindnera fabianii*, *S. cerevisiae* and *W. anomalus* were isolated (Monapathi et al., 2017). From antifungal susceptibility tests conducted, these isolates were resistant to commonly used antifungal agents. Some of the antifungal agents which the isolates showed resistance against, were also found in measurable quantities in the present study.

5.4. Conclusion

The presence of yeast in water has been related to pollution. In previous studies some of the isolated yeasts were pathogenic and when this water is used in direct contact scenarios, could result in infections. Results in this study have demonstrated that 26S rRNA gene sequences were detected at all sites in the Wonderfonteinspruit and Mooi Rivers. The Wonderfonteinspruit River sites had higher levels of the yeast marker genes compared to Mooi River sites. In the latter river, sites 6 and 7 (MR6 and MR7) had higher levels compared to the other sites. Measureable antifungal agents could be detected at the various sites. However, the Wonderfonteinspruit sites once again were most impacted. The latter results could potentially explain why antifungal resistant yeasts were frequently detected in previous studies.

The combination of environmental DNA analyzed by qPCR and/or NGS can be used to determine yeast pollution levels as well as the diversity of yeast rapidly. qPCR could also be used to target specific pathogenic species, providing rapid results on potential infection risks. This approach should, however, be optimized and standardized. Results from this study have demonstrated the potential of the analysis by qPCR. It is recommended that a NGS method should be developed to analyze eDNA for yeast diversity. Also, the development of specific qPCR assays for determining the presence of pathogenic yeast should be developed. This will be a very useful method that once standardized; could be employed to determine potential risk of infection by water users. Linking this to analyses of the same water for genetic elements (using the eDNA and qPCR) as well as chromatography approaches would be a powerful approach to study and monitor health risks associated with yeast polluted water.

CHAPTER 6

Conclusions and Recommendations

6.1. Conclusions

Water quality determines if water resources are fit for use for different activities (DWAF, 1996). The quality of the water is largely affected by effluents and discharges from industries, mines, wastewater treatment works and agricultural activities (Gupta et al., 2017). Criteria used, include routine monitoring of physico-chemical characteristics and microbiological pollutants in the water resources. Bacteria are normally included in these guidelines as microbial water pollution indicators. Yeasts have generally been disregarded in water quality analysis.

Water resources in South Africa are threatened by faecal pollution (Luyt et al., 2012). A large number (30.1%) of municipal WWTPs do not comply with regulated standards (DWAF, 2013a). In the NWP, there have been reports of untreated and poorly-treated sewage return flows entering surface water resources (DWAF, 2013a). WWTPs are important hosts to various human pathogens (Cai and Zhang, 2013). Consequently, when WWTPs effluents enter surface water resources, pathogens could also land in these resources (Lood et al., 2017). Pathogenic yeasts from clinical environments have been monitored and these are normally directly associated with infection/diseases (Ravikumar et al., 2015) in especially immunocompromised people. Limited information is available for pathogenic yeast species as well as antifungal agents in aquatic ecosystems. The present study was thus concerned with such aspects.

6.1.1. Aquatic yeasts and health implications: A review

The review paper addressed the presence of yeasts in surface water. The state of the surface water at large, the use and factors that compromise water quality were clearly described. These included how physico-chemical parameters are affected by human activities and may be the driving force to yeast levels and diversity in water. A broad description and identification methods for yeasts using morphological and physiological traits to molecular techniques were discussed. Useful aspects of yeasts as indicators of pollution were generally covered.

The review highlights the potential detrimental health effects that aquatic yeast species could have. Emphasis was on pathogenic and antifungal resistant yeasts as causal agents of diseases/infections especially in immunocompromised people. The review highlighted that the presence of pathogenic yeasts that are resistant to commonly used antifungal agents could cause deadly infections to water users. Virulence factors associated with pathogenic/opportunistic pathogenic yeast were also discussed.

6.1.2. Overview on water quality and yeast as indicators: Selected NWP surface water as examples

In aquatic environments, bacteria are largely studied. The present study is one of few yeast studies in freshwater environments. The part of the study was concerned with the correlation between physico-chemical parameters and yeast levels. It demonstrated that significant differences exist between physico-chemical parameters and yeast levels between different rivers and between sampling seasons. Most of the physico-chemical parameters were within the TWQR for livestock watering but not irrigation (DWAF, 1996). It was demonstrated that the Schoonspruit Rivers was the most polluted river system with

significantly high yeast levels and other parameters. A positive correlation was seen between some physico-chemical parameters and yeasts levels thus confirming that the parameters do influence yeast levels. Some of the identified species are regarded as pathogenic. A large proportion of the isolates were resistant to several of the antifungal drugs, particularly fluconazole. Immunocompromised people in the NWP who might use the water for direct purposes such as bathing, recreation etc. are likely to get infected by these yeasts. Antifungal resistance to these pathogenic yeasts could render treatment difficult when commonly prescribed antifungal drugs are used.

The major challenge in the present study was to determine the source of these antifungal resistant yeast as well as agents in water. They could be from human use, veterinary medicine or agricultural use since their mode of action and resistance mechanism is similar. The control of antifungal agents discharged into the environment requires strict intervention from the government and those in relevant authority. This would encompass proper and effective treatment, enforced legislature by various institutions and pharmaceutical companies that decant antifungals into the water.

6.1.3. Fluconazole resistance and resistance mechanisms in environmentally isolated *Candida albicans*

In this study, resistance mechanisms mediated by the presence of efflux genes were determined to explain fluconazole resistance in *Candida albicans*. Studies done to determine resistance mechanisms in *Candida albicans* to fluconazole have largely been conducted on molecular level in clinical isolates (Cernica and Subik, 2006, Sanglard et al., 2016). From antifungal susceptibility tests done in the present study, it was established that *C. albicans* isolates were resistant to fluconazole and several other antifungal agents.

The results of this study confirmed the presence of efflux pump genes in most ($\geq 60\%$) of *Candida albicans* isolates. The most frequently detected genes were *CDR1* and *CDR2*, followed by *FLU1* and subsequently *MDR1*. A phylogenetic analysis conducted between resistant genes of environmental and clinical sources in *Candida albicans* revealed a high gene sequence similarity. A high (99-100%) bootstrap confidence supported the relationship between the genes of environmental and clinical strains.

Environments that are polluted by discharges from antimicrobial users and manufacturers as well as WWTPs could mobilize transfer of resistance genes to human pathogens (Bengtsson-Palme et al., 2018). The presence of the genes in the present study confirmed that aquatic environments are possible harbour of the genes to human and animals. Water resources from which the *Candida albicans* isolates were retrieved are used for irrigation, recreational and religious purposes (NWP-SoER, 2014).

6.1.4. Perspective on pathogenic antifungals and yeasts in water using qPCR

In this chapter, a culture independent approach was used to indirectly determine yeast levels in water. Most yeasts studies employ culture dependant methods. Environmental DNA was extracted directly from the water and was subjected to qPCR. There are limited studies on the extraction of eDNA water as most studies have been done on wines. The PCR assay was efficient (slope= -3.23; correlation coefficient= 0.99; Bustin et al., 2009). Levels of yeasts expressed as copy number were quantified in all the sampling sites ranging from 2 to 18477 copies of yeast 26S DNA. End point PCR was positive in all sampling sites.

There are limited studies on pharmaceutical products as pollutants in aquatic environments. In the present study, antifungal agents were screened for (flucytosine, metronidazole, econazole, ketoconazole, miconazole and nystatin) using LC-MS-MS. All the antifungal agents except econazole and nystatin, were present in some of the sampling sites. Furthermore, fluconazole levels could be quantified and were present at 2 Wonderfonteinspruit River sites. These antifungal drugs could be originating from agricultural or urban sources. Pathogenic yeasts have previously been isolated from the same sampling sites and were resistant to commonly used antifungal drugs (Monapathi et al., 2017). The continuous exposure of environmental yeast to antifungal agents could potentially be responsible for observed antifungal resistance as noted by Monapathi et al. (2017).

The aim of the present study was to determine if there is interplay between water quality and antifungal levels as well as resistance of diverse yeast species from selected rivers in the NWP surface water. This was successfully demonstrated in the various chapters of this thesis.

6.2. Recommendations

- Stevens et al. (2003) suggested the use of other microorganisms, apart from bacteria as indicators of water pollution. Future studies on yeasts as aquatic microbiological pollution determinants should be conducted. The ability of heterotrophic microorganisms mostly bacteria to utilize organic matter in the water has led to studies addressing such. More studies on yeasts should be conducted as a complementary measure. Local and international regulatory authorities on water quality assessments should consider including yeasts in water quality guidelines. The related human risks of pathogenic yeasts in water will be addressed in such interventions. The guidelines will be helpful in responding to some of the diagnostic yeast infections experienced from direct contact with polluted surface or drinking water.
- Apart from temperate surface water, other geographical areas should be considered. These include tropical and extreme environments. Studies from these environments will provide us with in-depth knowledge on the yeast diversity and functionality with relevance to varying climates and environmental conditions. It will also provide insights into whether such diversity could be associated with human health risks.
- It was demonstrated in the present study that surface water was polluted with yeasts and antifungal agents. However, the source of the cause of pollution could not been established. For future research, it is imperative to identify the point sources and non-point sources of pollution. This will assist with mitigation measures as well as policy formulation to put measures in place to reduce pollution. The polluter pays principle can be enforced. Moreover, the source of pathogenic

microbes and antimicrobial resistance genes could be established and further pollution can be prevented.

- Most studies that determine mechanisms of antifungal resistance are conducted in *Candida albicans* and resistance to fluconazole. Many of these studies were conducted on clinical isolates. For future studies, more pathogenic non-albicans *Candida* species should be studied and other antifungal agents should be incorporated. The present study has shown high phylogenetic sequence similarity between efflux pump resistant genes in environmental and clinical isolates. This is beneficial to explain antifungal resistance that could be experienced when environmental yeast infections are considered. More molecular based resistance mechanisms including those mediated by mutations should also be explored. Molecular studies on antifungal resistance will assist in developing new ways and strategies to overcome worldwide antimicrobial resistance crisis.
- Gene Expression of antifungal resistance genes in pure yeast isolates should be determined in the future. The presence of these genes from conventional PCR based technique does not essentially infer that they are expressed. Quantitative PCR and/or western blot analysis should be conducted to determine the actual *in vivo* expression level of these genes.
- DNA barcoding and other housekeeping genes may provide some interesting markers to detect pathogenic species using qPCR approaches. Furthermore, 26S rRNA gene and next generation sequencing (NGS) has provided diversity and functional dynamics of yeasts. There are limited studies on aquatic yeasts. NGS studies should be conducted on aquatic yeast using the 26S rRNA gene to gain overall insights into diversity.

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